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# THE GENUS SHIGELLA

## (DYSENTERY BACILLI AND ALLIED SPECIES)

ERWIN NETER

*Department of Bacteriology and Immunology, University of Buffalo, School of Medicine and  
Laboratories of the Children's Hospital, Buffalo, New York*

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In 1898, Shiga first proved that dysentery of man may be caused by a bacillus, and thereby established the etiological distinction between bacillary and amebic dysentery. During a severe outbreak of dysentery in Japan, he isolated a bacillus, now called *Shigella dysenteriae* (Shiga), that was specifically agglutinated by the sera of patients with dysentery. In the two decades following this discovery, other bacilli were recovered from patients with epidemic or endemic dysentery and shown to be the causative agents of this disease. They differed, however, in certain respects from *S. dysenteriae* and are classified today as *S. paradysenteriae*, *S. sonnei*, and *S. schmitzi*. These four species have certain essential features in common and are considered, therefore, as members of one group—the genus *Shigella*. They are, furthermore, recognized as the cause of bacillary dysentery of man (true dysentery bacilli).

Other microorganisms which share certain morphological, cultural, biochemical, and antigenic characters with the true dysentery bacilli, and are, therefore, justifiably included in the genus *Shigella*, are not associated with epidemic or endemic bacillary dysentery of man. Although pathogenicity or lack of it is of prime importance to the medical bacteriologist, this characteristic alone is not a sound basis for the classification of bacteria. Unfortunately, the members of this genus which lack a relation to bacillary dysentery and, therefore, are

often considered to be non-pathogenic, have not received the same attention as have the true dysentery bacilli. Mention should be made, however, that some members of the genus which are not associated with dysentery, nevertheless, may be pathogenic for human beings and animals and cause diseases other than dysentery. Moreover, from the viewpoint of systematic bacteriology these members are as important as the true dysentery bacilli. Therefore, a review of the genus *Shigella* has to deal not only with those members that cause epidemic or endemic bacillary dysentery, but also with those species that are not specifically associated with this disease, that is, the allied species of Andrewes (7). The attempt is made here to present the pertinent data of the genus as a whole and to discuss certain features of the different species belonging to it.

#### THE GENUS SHIGELLA AS A WHOLE

It is rather difficult, at the present time, to give a definition of the genus *Shigella* which is sufficiently distinctive to differentiate it from others and yet broad enough to include all of its members. Moreover, it is safe to assume that some species now included in this genus may be eliminated and that others may be added in the future.

The members of the genus *Shigella* are commonly regarded as gram-negative, non-acid-fast bacilli, that have no spores and no capsules. The morphology of the various members offers no means of differentiation either from each other or from other non-motile, non-encapsulated, gram-negative bacilli of the tribe *Salmonelleae*. It is generally agreed that, with the possible exception of *S. sp.* (Newcastle type), whose taxonomic position is not as yet definitely established, the species of *Shigella* are non-motile and do not possess flagella.

In regard to the cultural characters, it may be pointed out that the majority of the members grow well on the usual culture media (including Endo agar) and are easily maintained thereon over long periods of time. They are aerobic, and most of them facultative anaerobic bacilli. However, it is important to note that certain species now included in the genus *Shigella* differ in their cultural characters. *S. septicaemicae*, for instance, thrives only in the presence of oxygen and fails to grow on Endo agar. These and other exceptions are generally overlooked in presentations of the genus as a whole.

Like many other bacteria, the members of the genus *Shigella*, too, produce different forms of colonies on agar plates. Unfortunately, dissociation has not always been adequately considered even in recent literature, and many contradictory statements can be found in regard to reversion among the different cultural phases, the sequence of phase transformation, and association of these phases with other characters of the microbes such as biochemical attributes, antigenic structure, and virulence. Arkwright (9) has described in detail the S and R forms of *S. dysenteriae* (Shiga). The former colonies are smooth, round, and domed and the latter flattened with irregular margins and surfaces. The smooth colonies emulsify without auto-agglutination in physiological salt solution, whereas the rough colonies show clumping. In addition to these smooth and rough colonies, intermediate colonies (Rs, RS, rS forms) are fre-

quently observed. The mucoid phase (M form), that occurs in many bacterial species, has been encountered also in members of the genus, for instance, in *S. equirulus*. Edwards (56) deserves credit for this important observation. Minute forms (dwarf or D colonies, G colonies<sup>1</sup> of Hadley (79) and G-like forms, *Zwergholonien*) of certain bacterial species have been repeatedly reported both by European and American bacteriologists. Such forms have also been described among members of the genus *Shigella*, particularly, *S. dysenteriae*, *S. sonnei*, and *S. equirulus*. Dissociation and the various phases of bacterial growth among members of this genus, so far as they occur, will be considered with the description of the different species. This seems preferable to a general discussion, because dissociation in certain species of the genus has not as yet been studied adequately, and findings in one species do not necessarily hold true in others.

With respect to their biochemical activities, the members of the genus *Shigella* have but few reactions in common. Those members which are associated with epidemic or endemic bacillary dysentery of man (with the exception of *S. sp* (Newcastle type)) produce acid but no gas from glucose, cause acid formation in litmus milk, reduce nitrates to nitrites,<sup>2</sup> form  $\text{NH}_3$ , are Voges-Proskauer-negative, fail to form  $\text{H}_2\text{S}$ , do not grow in Koser's citrate medium, and do not liquefy gelatin (181). Some microorganisms now included in the genus, however, differ in their characters from the above, for instance, *S. sp* (Newcastle type) produces acid and gas from glucose, *S. septicaemiae* liquefies gelatin, and so forth. Furthermore, it should be pointed out that some of the members of this genus have not been studied adequately in the past. For these reasons, any attempt to present the basic and common biochemical characters of the genus *Shigella* is beset with the greatest difficulties. Tables 1, 3, 4, 7, and 8 give the more important biochemical characteristics of the various members, because, at the present time, this seems more profitable than any such treatment of the genus as a whole.

Attention may be called to the appearance of variants having increased biochemical activity. The Sonne dysentery bacillus serves perhaps as the best example. On agar plates papillae or daughter colonies frequently appear which consist of raised, smooth, entire, rounded outgrowths on the surface. Characteristically, these daughter colonies, in contrast to the parent colonies, ferment lactose. The appearance of these papillae upon prolonged incubation seems to be responsible for the late fermentation of lactose rather than a slow utilization of this carbohydrate.

During the last few years our knowledge of the growth requirements of many microorganisms has increased considerably, although many questions remain unanswered. It is interesting to mention that important information on this subject has resulted from investigations on the mode of action of the sulfonamides. Until recently, little was known in regard to the more specific growth require-

<sup>1</sup> In this review, the term G colony is not restricted to those composed of filtrable elements alone.

<sup>2</sup> Some authors (149) differ on the question of reduction of nitrates.

ments of the various species of the genus *Shigella*. The observations of Koser and his associates (102, 50), therefore, are of particular interest. These authors found that many Flexner and Sonne dysentery bacilli either failed to grow or grew only poorly in a basic medium consisting of 15 amino acids, glucose and inorganic salts. Addition of nicotinamide or nicotinic acid exerted a marked growth-promoting effect. These substances, therefore, may be considered as essential growth factors for these strains. In passing, it may be mentioned that the stimulation of respiration due to nicotinamide is inhibited by sulfapyridine (51). It will be of interest to see whether the other species of the genus have similar or different growth requirements.

Whether or not the genus *Shigella* is characterized by an antigenic component which is common to all its members and differentiates it from other genera (genus-specific antigen) can not be definitely answered at the present time, particularly, since the antigenic structure of some of the members of the group has not as yet been thoroughly investigated. It is highly desirable to know whether or not such a genus-specific antigen exists, its demonstration would aid greatly in the identification of unknown strains and in the classification of species whose taxonomic position has not been firmly established. A discussion of the antigenic structure of the more important members of the genus will be presented below.

A primary subdivision of the genus *Shigella* is best obtained by classifying its members according to their action upon mannitol and lactose. Thus, four main groups are obtained, whose more important members are presented in table 1. As outlined in the introduction, the genus is further subdivided into true dysentery bacilli and allied species according to the relation of the respective species to epidemic and endemic bacillary dysentery of man. Fortunately, this primary subdivision of the genus according to the biochemical characters of its members is compatible with the equally important classification based upon the antigenic structure.

The members of the genus which, at the present time, are recognized as the cause of epidemic and endemic dysentery in man, are *S. dysenteriae* (Shiga), *S. schmitzi*, *S. sp.* (Newcastle type), *S. paradysenteriae*, and *S. sonnei*. Unfortunately, these true dysentery bacilli are referred to in the literature under a variety of names. Table 2 presents a comparative classification and the respective terminology of different authors.

A few facts pertinent to the bacteriological and immunological aspects of bacillary dysentery may be mentioned. Bacillary dysentery of man, characterized by a more or less severe inflammation of the large intestine, may be epidemic, endemic, or sporadic. It may occur spontaneously also in monkeys and dogs. Sometimes, acute bacillary dysentery of man is not terminated by recovery, but continues as a subacute or chronic colitis. However, it has not as yet been conclusively shown that dysentery bacilli or other intestinal microorganisms play a significant pathogenic role in this form of colitis.

The bacteriological diagnosis of bacillary dysentery is based upon the isolation of dysentery bacilli from the feces. This may be carried out successfully

in a relatively high percentage of cases, provided that the bacteriological examination is done in the first few days of the disease and appropriate methods are

TABLE 1

*A primary classification of the members of the genus Shigella*

	GROUP I LACTOSE NEGATIVE MANNITOL NEGATIVE	GROUP II LACTOSE NEGATIVE MANNITOL-POSITIVE	GROUP III LACTOSE POSITIVE MANNITOL-NEGATIVE	GROUP IV LACTOSE POSITIVE MANNITOL-POSITIVE
True dysentery bacilli	<i>S. dysenteriae</i> <i>S. schmitzi</i>	<i>S. paradysenteriae</i>		<i>S. sonnei</i>
Allied species	<i>S. ambigua</i> <i>S. septicaemiae</i> <i>S. minutissima</i>	<i>S. alkalescens</i> <i>S. gallinarum</i> <i>S. pfaffii</i> (?) <i>S. rettgeri</i> (?)	<i>S. gintottensis</i> <i>S. bienstockii</i> (?) <i>S. oxygenes</i> (?)	<i>S. equiruli</i> <i>S. ceylonensis</i> B <i>S. madampensis</i>

TABLE 2

*Comparative classification of the true dysentery bacilli according to different authors\**

BERGEY, ET AL.	ANDREWES AND INMAN	KRUSE	LENTZ AND PRIGGE	SONNE	AOKI
<i>Shigella dysenteriae</i> (Shiga) Castellani and Chalmers	<i>Bacillus</i> <i>Shiga</i>	True dysentery bacillus	Shiga-Kruse bacillus	Shiga Kruse bacillus	Group VIII
<i>Shigella ambigua</i> (Andrewes) Weldin	<i>Bacillus</i> <i>ambiguus</i>	Pseudo-dysentery bacilli I and J	Schmitz bacillus		
<i>Shigella paradysenteriae</i> (Collins) Weldin†	<i>Bacillus</i> <i>Flexner</i> — Y	Pseudo-dysentery bacilli			
	Y V (*)	B	Flexner bacillus	Group II	
	VZ	A			
	Y	D	Y bacillus (Hiss Russel)	Group I	Group I
	Z	H			Group X
	WX X	C F			Groups II III, IV, V (?)
	W (?)	G	Strong bacillus	Group II	
<i>Shigella sonnei</i> (Levine) Weldin	<i>Bacillus</i> <i>dispar</i>	Pseudo-dysentery bacillus E	Kruse-Sonne bacillus	Group III	

\* Compiled after Lentz and Prigge (109)

† See also Table 6

employed. In the majority of cases of bacillary dysentery the causative microorganisms remain localized in the intestinal tract and in the regional lymph glands. In some cases the dysentery bacilli may be found in the feces in almost

pure culture, in many cases, however, they represent only a small percentage of all the colonies cultivated, or, they may not be isolated at all. In the latter instance, the possibility should be considered that bacteriophage may be present in the feces. The demonstration of bacteriophage may aid in the diagnosis of bacillary dysentery (61, 190).

Invasion of the blood stream by dysentery bacilli is rare, although this may occur (152, 90, 90a, and others). Occasionally, the true dysentery bacilli such as the Shiga, Flexner, and Sonne bacilli may invade other organ systems and cause infections, for example, of the urinary tract (152, 137, 138, 142).

The technical procedures used for the isolation of dysentery bacilli from the feces will be mentioned but very briefly. It is important to examine the feces as soon as they are obtained, particles containing pus and blood are suspended in physiological saline solution or in infusion broth. When immediate examination of the specimen is not possible, the material can be preserved best in a buffered saline solution (pH 8.5) containing 1% sodium citrate and 0.5% sodium desoxycholate (16). This procedure deserves more widespread use in hospital laboratories. It is advisable to seed several agar plates with the suspension and to use a number of differential culture media such as Endo agar, MacConkey agar, eosin-methylene-blue-lactose agar, desoxycholate-citrate agar, lithium chloride-Endo agar, or others. Bismuth sulfite agar, that is so successfully used for the isolation of typhoid bacilli, is not suitable for the recovery of dysentery bacilli.\* Recent studies indicate that desoxycholate-citrate agar allows heavier seeding than MacConkey agar and gives superior results (122, 86, 93a, and others). Inoculation of the plates is carried out in this laboratory as follows: a small area (approximately one-third) of each plate is first seeded, then, the loop is sterilized and cooled, and material from the first inoculation of the plate is taken up and streaked on another part of the same plate (second third), material from the second inoculation is then taken up for seeding of the remaining part of the plate. Thus, isolated colonies are usually obtained. After incubation for 18 to 24 hours at 37 C non-lactose-fermenting colonies are subcultured. Fishing of lactose-negative colonies after further incubation may increase the percentage of successful isolations (122). These colonies are then studied in regard to morphology, motility, cultural characters, biochemical activities, and antigenic structure. In this laboratory, fermentation tubes are used with glucose, mannitol, and dulcitol. Since autoclaving at 15 pounds causes a partial breakdown of some test substances, thus leading to false reactions, it is advisable to sterilize broths containing lactose, maltose, sucrose, rhamnose, and xylose by filtration through Seitz filters. Autoclaving may not produce a noticeable change in pH, however, organisms which do not ferment the original substances may act upon the breakdown products with the formation of acid, or acid and gas. Such reactions must be carefully avoided.

\* Very recently, Wilson and Blair (192a) described a new culture medium selective for Flexner dysentery bacilli. On this tellurite-iron-rosolic acid agar Flexner dysentery bacilli grow profusely, whereas *E. coli* and *A. aerogenes* are largely inhibited. Thomas and Hulme (189a) have confirmed the usefulness of this medium.

For diagnostic agglutination tests sera of immunized rabbits are preferable. When antisera of horses are employed, it should be kept in mind that the titer of normal agglutinins is relatively high and certainly above that of rabbit and human sera.

The serological diagnosis of bacillary dysentery (Widal test) presents greater difficulty by far than does that of typhoid fever, this is particularly true in cases with infections due to members of the Flexner group. Whenever the serological diagnosis of infections due to species of *Shigella* is carried out, it is essential (a) to use standardized suspensions of known agglutinability, (b) to employ a standardized procedure (temperature, time of incubation, etc.), (c) to know the titer of normal agglutinins for the respective suspensions, and (d) to employ proper positive and negative controls. For the diagnosis of mixed infections with two or more species of *Shigella*, the Castellani test may be used successfully. The uncritical employment of the Widal test for the diagnosis of bacillary dysentery has, unfortunately, done much to discredit this method, some authors, for example, have postulated a certain titer of agglutinins for *all* types of dysentery bacilli as diagnostic of previous or present infection without due consideration of the points just outlined.

The following therapeutic sera have been used successfully in the treatment of bacillary dysentery of man and of experimental infections of animals: (a) Immune sera containing antibodies directed against the exotoxin of Shiga's bacillus (anti-exotoxin), and (b) immune sera containing antibodies directed against the dysentery bacilli themselves or their endotoxins (immune-opsonins or tropins, bactericidal antibodies and anti-endotoxins) (65, 19a). Human convalescent serum has also been recommended (63).

A second class of specific agents for the treatment of such infections is bacteriophage. There is a considerable literature on the subject, but it is not yet possible to make a final statement as to the efficacy of this treatment. Certain authors believe that the presence of highly effective bacteriophage may terminate the disease and that its therapeutic application is of definite value (91, 182, 160, and others). Others, however, feel that the oral administration of bacteriophage does not alter the clinical course of acute bacillary dysentery (155).

Much more promising than treatment with either bacteriophage or antibacterial immune serum are chemotherapeutic compounds of the sulfonamide type. Space does not permit a detailed discussion, a few data, however, may be given. Certain sulfamido compounds are definitely bacteriostatic toward dysentery bacilli. Moreover, Levaditi and Vaisman (110) demonstrated that sulfanilamide and related compounds prevent the effects of certain dysenteric endotoxins *in vivo*. However, the role of this 'anti-endotoxic' action of sulfonamides in the treatment of spontaneous *Shigella* infections of animals and man remains to be determined. Sulfathiazole and sulfaguanidine have proved to be efficacious in the treatment of experimental infections of animals and of bacillary dysentery of man (Marshall and associates (121), Weil and Gall (187), Lyon (115), Cooper and Keller (44, 45), and Libby (114)). Sulfaguanidine and similar compounds seem to be particularly suitable for the treat-

this disease, since they inhibit the growth of members of the genus *Shigella*, are fairly soluble in water, and are only poorly absorbed from the intestinal tract. Thus, relatively high concentrations of the drugs can be obtained at the site of the infection.

In regard to specific prophylaxis of bacillary dysentery, active immunization has been attempted by means of (a) Shiga exotoxin-antitoxin mixtures, (b) Shiga toxoid, and (c) killed bacterial suspensions. With war raging in parts of the world where bacillary dysentery is endemic, the prevention of this disease by means of active immunization is of particular interest now and will be further discussed below.

#### GROUP I THE LACTOSE-NEGATIVE, MANNITOL-NEGATIVE MEMBERS

1 *S. dysenteriae* (Shiga) Castellani and Chalmers is the type species of the genus *Shigella*. It is a gram-negative, non-motile bacillus, it has no capsule and forms no spores. It grows well on ordinary culture media, the optimal temperature for its cultivation is about 37°C. Upon incubation for 24 hours on plain agar or MacConkey agar colonies in the S phase are round, shiny, smooth, sharply defined, domed, and translucent. Colonies in the R phase may be either flat and thin with irregular outlines and rough surfaces, or they may be moderately thick, but flattened, with indented or slightly jagged margins. Generally, after 24 hours of incubation, the R forms of Shiga's bacillus are somewhat larger than the S forms. In addition to typically smooth and rough colonies, intermediate forms are frequently seen. R variants may develop from S strains spontaneously, particularly in old cultures, or under a variety of different conditions, for instance, in the presence of bacteriophage Arkwright (9), whose investigations of the different cultural forms of the Shiga dysentery bacillus are of outstanding importance, found that changes of the culture phase are associated with changes of other attributes. The S forms give stable suspensions in physiological salt solution and cause uniform turbidity in broth cultures. In contrast, the R forms agglutinate spontaneously in physiological salt solution and form a deposit in broth cultures. Reason for this different behavior of S and R forms of *S. dysenteriae* is of interest. White (191) found that agglutination of the R form of Shiga's bacillus in physiological salt solution can be prevented, if the alcohol-soluble substances of the bacilli are previously removed. It seems desirable to investigate the nature of these alcohol-soluble substances. S and R forms differ also in their antigenic structure, as will be discussed below. In addition to these normal sized colonies, cultures of Shiga's bacillus may occasionally show very small colonies, the so-called G or G-like colonies. There cannot be any doubt about the existence of this colony form. On the other hand, it remains to be seen whether or not elements of these colonies are filtrable. Attempts to confirm the original observations (80) of a filtrable variant of *S. dysenteriae* have not been successful (79). This, however, should not be interpreted to indicate that filtrable forms of bacteria in general do not exist. This particular phase of the problem of bacterial dissociation will be discussed further in the section on *S. sonnei*.

The biochemical reactions of the Shiga bacillus are very characteristic. They are presented in table 3. As may be seen from table 3, *S. dysenteriae* differs in its biochemical reactions from the other members of the group. Its failure to form indole clearly differentiates the Shiga from the Schmitz bacillus, the other member of the group which may cause dysentery of man.

Antigenically, the R and S forms of the Shiga dysentery bacillus are distinct. This was shown by Arkwright (9) in 1921. Failure to correlate bacterial dissociation and accompanying changes in the antigenic structure of the Shiga bacillus accounts for many reports in the literature on so-called inagglutinable strains. Arkwright showed that rabbits immunized with pure phases of either R or S forms develop agglutinins against the homologous organism, these antisera give only very little cross-reaction with the heterologous organism.

TABLE 3

*Biochemical reactions and antigenic structure of the lactose-negative, mannitol-negative group*

	INDOLE	LITMUS MILK	GLUCOSE	LACTOSE	SUCROSE	MANNITOL	MALTOSE	REAMONG*	XYLOSE	DULCITOL	ANTIGENIC STRUCTURE
<i>Shigella dysenteriae</i> (Shiga) Castellani and Chalmers	-	A-Alk	A	-	-	-	-	-	-	-	Homogeneous
<i>Shigella schmitzi</i> , Hauduroy, et al	+	A	A	-	-	-	- occas A	A	-	-	Homogeneous
<i>Shigella ambigua</i> (Andrews) Weldon	+	A-Alk	A	-	- occas A	-	-	A	-	-	Incompletely studied
<i>Shigella septuacemias</i> (Bergey, et al) Bergey, et al	+	A or -	A	-	-	-	-	-	-	-	Unknown
<i>Shigella minutissima</i> (Migula) Bergey, et al	-	AC	A	-	-	-	-	-	-	-	Unknown

A = Acid reaction

C = Coagulation

Alk = Alkaline reaction

± = Indole production

- = No indole or acid production

\* Synonym for isodulcitol

The S and R forms of all strains are agglutinated by the respective antiserum. It is now generally agreed that in its smooth phase this species is antigenically homogeneous. Arkwright noted, furthermore, that the S form is agglutinated in large clumps by specific antiserum, whereas the R form is agglutinated in small clumps which are readily shaken up into a turbid suspension. Absorption experiments give further evidence of the distinct antigenic structure of these two cultural phases. Absorption of a polyvalent serum (which agglutinates both R and S forms) with the S form eliminates the antibodies to the latter without reducing to any great extent the titer of the antibodies to the R form, and *vice versa*, the R form absorbs its own agglutinins from such a serum without markedly reducing the titer of the antibodies to the S form.

The chemical analysis of the various antigenic substances, which give the characteristic immunological pattern to bacterial species and their variants, has yielded interesting information in the past. Such investigations of Shiga's dysentery bacillus have been particularly fruitful at the hands of Morgan and his associates (129, 130). Similar studies on other species of the genus *Shigella* are highly desirable.

The antigen in smooth strains of the Shiga bacillus which gives them their species-specificity has been isolated and purified (129, 130, 150). This antigen consists of three main components: (a) a polysaccharide, (b) a polypeptide-like substance, and (c) a phospholipin. The latter seems to be of minor significance. The polysaccharide absorbs not only the agglutinins to the Shiga bacillus, but also the heterophile antibodies, which may be induced by the injection of the whole antigen-complex or of intact bacilli. This heterophile hapten of the Shiga bacillus was first demonstrated by Iijima (93), who found that rabbits injected with a suspension of Shiga's bacillus formed sheep-cell hemolysin of the Forssman type. According to the investigations of Meyer and Morgan (124, 125), the formation of these antibodies is induced not by two different substances, but by a single polysaccharide with two distinct reactive groups: one reacting with the agglutinin to the Shiga bacillus, the other with the sheep-cell hemolysin. This observation is of great general immunological interest. The polysaccharide is a typical hapten; alone, it does not induce the formation of antibodies *in vivo*. The polysaccharide-polypeptide mixture engenders antibodies against both components (150). It is interesting to note that antibodies against the polypeptide are not present in immune sera prepared by the injection of the organisms themselves. The significance of these antibodies in immunity remains to be determined. All strains of the Shiga dysentery bacillus do not contain the heterophile sheep-cell hapten. Shiga's bacillus may lose the sheep-cell hapten upon treatment with bacteriophage (28). The presence or absence of this heterogenetic sheep-cell hapten depends not only upon the individual strain, but also upon the composition of the culture medium (60). It is evident that any such changes in antigenic structure should be carefully correlated to possible changes of the culture phase of the organism. This, however, has not always been done in the past. In addition to the species-specific bacterial polysaccharide, the Shiga bacillus may contain other antigenic components, for instance, antigens in common with human blood cells (58, 59, 162). The significance of the Forssman and blood-group antigens in Shiga's bacillus and, particularly, their relationship to pathogenicity, remain to be determined.

The chemical structure of the antigen or antigens characteristic of the R phase of the Shiga dysentery bacillus is not known as yet.

Levine and Frisch (112) and Burnet (31 to 34) made the important observation that a relationship exists between the effectiveness or ineffectiveness of isolated strains of bacteriophage and the antigenic structure of the respective bacterial strain. Arkwright (10), in 1924, reported that from S forms of Shiga's

bacillus, which are resistant to bacteriophage, phage-susceptible variants may be obtained. It is interesting to note that these variants are always R forms.

A few years following the discovery of the Shiga dysentery bacillus, it was found that sterile culture filtrates were toxic for rabbits, causing diarrhea, paralysis of the extremities (due to lesions in the central nervous system) and death (43, 134, 158). The exotoxin of *S. dysenteriae* is often referred to as a neurotoxin, because it affects the nervous system. It is highly reactive in rabbits and horses and less so in mice, guinea-pigs, and rats. It may be obtained by filtration of broth cultures, an incubation for two weeks yielding smaller amounts of exotoxin than incubation for three to four weeks. The exotoxin may also be obtained from the bacterial cells themselves for instance, by repeated freezing and thawing, by shaking, by chemical methods, and also by the action of bacteriophage. The latter procedure was used successfully by Kuhn (106). It is generally true that, so far as members of the enteric group of bacilli are concerned, strains in the S phase are more toxic than in the R phase. The observation of Thibault and Braunberger (178) who reported that smooth and rough colonies of a Shiga strain were equally toxic, thus requires confirmation.

During recent years, several attempts have been made to separate the exotoxin of the Shiga bacillus from the endotoxin. Olitsky and Kligler (146), Boivin and Mesrobian (20 to 22), and Haas (76) claim to have accomplished this separation. These authors report, furthermore, that the exotoxin affects the nervous system (neurotoxin), whereas the endotoxin causes lesions of the intestinal tract (enterotoxin). This opinion, however, has not been generally accepted (184).

Formaldehyde exerts a characteristic influence upon the exotoxin in reducing its toxicity without diminishing its antigenic properties to any great extent. The formaldehyde-treated exotoxin (toxoid) of Shiga's bacillus is not completely devoid of toxicity (83). The toxoid may be successfully used for the immunization of animals in order to obtain high-titered antisera for therapeutic purposes. The mortality rate of the animals injected with toxoid is markedly lower than that of the animals immunized with the untreated toxin. This toxoid possibly may be employed also for the active immunization of man and may prove valuable now, with war raging in areas where Shiga bacillus infections are endemic.

Cultures of the Shiga dysentery bacillus yield a filtrable substance of toxin-like nature which elicits the so-called Schwartzman phenomenon (167). An effective filtrate is obtained by growing a suitable strain on agar surface, washing the growth off with physiological salt solution and passing the fluid through a Berkefeld filter. When such a filtrate is injected into the skin of rabbits, followed 24 hours later by an intravenous injection of the same or certain other filtrates, a severe hemorrhagic-necrotic lesion appears at the site of the first injection. In spite of the fact that this phenomenon has been investigated extensively in animals, the role of the Schwartzman factor in the pathologic

of bacillary dysentery of man is by no means clear, especially since it is not even known whether this substance is produced under natural conditions of infection

Brief mention may be made of the reported existence in Shiga bacilli of substances which induce allergic reactions in sensitive individuals (169) The nature of these allergens and their significance in bacillary dysentery of man remain to be determined

2 *S schmitzi* Hauduroy, *et al* 3 *S ambigua* (Andrewes) Weldin In 1917, Schmitz (164) isolated from patients with clinical signs of dysentery a bacillus which showed certain similarities to Shiga's bacillus and yet revealed striking differences A similar microorganism was studied by Andrewes (7) in 1918, who referred to it as *Bacillus ambiguus* At the present time, it is impossible to decide whether all organisms identified as either *S schmitzi* or *S ambigua* are identical or differ in some as yet unknown properties Not until these strains have been studied adequately, will it be possible to state whether these organisms form one single or two different species It is true that Schmitz's bacillus has been recognized as a cause of bacillary dysentery (164, 163), and that Andrewes' bacillus was considered to bear no relationship to dysentery of man In this connection it may be pointed out that the factors responsible for pathogenicity of certain strains and species are still very incompletely known Furthermore, it has not been explained why one species (*e g*, *S paradysenteriae*) may cause epidemic outbreaks of dysentery in man and a very closely related species (*e g*, *S alkalescens*) does not In the latest edition (1939) of Bergey's Manual of Determinative Bacteriology (19) and in A System of Bacteriology in Relation to Medicine (72) only one species is recognized An extensive study of all features of these microorganisms is definitely needed, and it should be kept in mind that such closely related organisms as *S paradysenteriae* and *S alkalescens* are recognized today as two distinct species

*S schmitzi* resembles Shiga's bacillus, but it may readily be distinguished from the latter by its capacity to form indole and to produce acid from rhamnose (table 3) Antigenically, this species is homogeneous (161) and distinct from Shiga's bacillus Antisera to the Schmitz bacillus obtained from rabbits agglutinate Shiga's bacillus only slightly or not at all Likewise, antisera to Shiga's bacillus fail to agglutinate to high titer the bacillus of Schmitz When cross-reactions are encountered, agglutinin-absorption tests allow a definite differentiation of the two organisms Another important difference between the two species exists with respect to their toxicity, the Shiga bacillus producing a powerful exotoxin, while the other does not

4 *S septicaemiae* (Bergey, *et al*) Bergey, *et al* 5 *S minutissima* (Migula) Bergey, *et al* Both of these microorganisms were first described many years ago and have received but little attention recently Only a few data are available A thorough reinvestigation of these two species is definitely needed Otherwise, the incomplete information will be carried from edition to edition of the manuals of bacteriology Only when the characters of these organisms are known adequately, will it be possible to identify unknown strains, to study

the incidence and pathogenicity, and to reconsider the taxonomic position of these two species

*S. septicaemiae* was isolated in 1904 by Riemer (156) from geese and referred to as "*Bacillus septicaemiae anserum exsudativae*" It is a small rod (0.1 by 0.3 to 1.0  $\mu$ ) According to Riemer, it thrives only in the presence of oxygen and thus differs from other members of the group which are facultative anaerobes Apparently, the growth requirements of this organism differ from those of the majority of the members of the genus *Shigella*, inasmuch as *S. septicaemiae* fails to grow on Endo agar In a medium containing glucose it causes a change of the pH to the acid side It fails to ferment lactose and mannitol (table 3), and may form  $H_2S$  and traces of indole It liquefies gelatin

*S. minutissima* was first isolated in 1896 by Kruse (104) from an abscess and described as "*Bacillus pyogenes minutissimus*" It is a small rod (0.5 by 1.0  $\mu$ ) It produces acid from glucose, but not from mannitol and lactose (table 3) It causes acid and clot formation in litmus milk It fails to produce indole and does not liquefy gelatin

6 *S. sp* (Newcastle type) In 1929, Clayton and Warren (41, 42) isolated from patients presenting symptoms of dysentery a microorganism which they considered to be the cause of the disease and which differed from previously described species Since then, this species has been encountered also in the United States

At the present time, this microorganism is regarded as a member of the genus *Shigella* (19) It must be pointed out, however, that this bacillus differs in certain characters from all other members of this genus In the first place, it is considered to be somewhat motile In the second place, it produces small amounts of gas from certain carbohydrates in peptone-water and even larger amounts when grown in Lemco-broth Its taxonomic position, therefore, has to be reconsidered The fact that this microorganism may cause a dysentery-like disease in man, is not sufficient reason to include it in the genus *Shigella*

*S. sp* (Newcastle type) produces acid and gas from glucose, maltose, and dulcitol, it fails to attack lactose, sucrose, and mannitol, it causes acid formation in litmus milk and does not produce indole Strains similar to the Newcastle bacillus, but fermenting mannitol have been described (52)

Antigenically, this species is homogeneous, according to the investigations of Clayton and Warren (41, 42) It is interesting to note that the Newcastle bacillus bears no antigenic relationship to Shiga's bacillus, but gives cross-reactions with certain members of the Flexner group It may be mentioned in this connection that patients with dysentery due to *S. sp* (Newcastle type) may develop specific agglutinins Normal human sera fail to agglutinate this organism

#### GROUP II THE LACTOSE-NEGATIVE, MANNITOL-POSITIVE MEMBERS

7 *S. paradysenteriae* (Collins) Weldin is often referred to as the Flexner group of dysentery bacilli As a typical member of the genus *Shigella*, it is a non-motile, gram-negative bacillus Its average size from mature agar

cultures is 0.5 by 1.0 to 1.5  $\mu$ . It grows readily on ordinary culture media, including Endo agar. On plain agar the colonies may have the typical features of S, R, or SR forms. The R forms may occur spontaneously, particularly in old cultures, or they may be obtained by growing S forms in broth containing homologous antiserum, bacteriophage, or certain disinfectants such as phenol or formaldehyde. Papillae are less frequently seen with members of the Flexner group than with Sonne's bacillus. On lactose-containing media these papil-

TABLE 4

*Biochemical reactions and antigenic structure of the lactose-negative, mannitol-positive group*

	INDOLE	LITMUS MILK	GLUCOSE	LACTOSE	SUCROSE	MANNITOL	MALTOSE	REAMNOSE	XYLOSE	DULCITOL	ANTIGENIC STRUCTURE
<i>Shigella paradysenteriae</i> (Collins) Weldin	+ occas —	A-Alk	A	—	— or A	A	A or —	—*	—	—*	13 or more types
<i>Shigella alkalescens</i> (Andrews) Weldin	+	A-Alk	A	—	— occas A	A	A	A	A	A	2 types
<i>Shigella gallinarum</i> , (Klein) Weldin	—	— (Alk ?)	A	—	—	A	A	A	A	A	Homogeneous (0- antigen of Group D of genus <i>Salmonella</i> )
<i>Shigella pfaeffii</i> (Hadley, et al.) Weldin	—	—	A	—	—	A	A	A	A	—	Incompletely studied
<i>Shigella rettgeri</i> (Hadley, et al.) Weldin	— (+ ?)	Alk	A	—	—	A	—	?	A	—	Incompletely studied

A = Acid reaction

+ = Indole production

Alk = Alkaline reaction

— = No indole or acid production

\* On prolonged incubation, acid may be produced by some strains

TABLE 5

*Old classification of the varieties of Shigella paradysenteriae according to sugar fermentations*

VARIETY	GLUCOSE	MANNITOL	MALTOSE	SUCROSE
Flexner	A	A	A	—
Flex	A	A	—	—
Strong	A	A	—	A

A = Acid production, — = no acid production

lae remain lactose-negative (74). On media containing other carbohydrates such as maltose and sucrose, these daughter colonies may show increased fermenting power.

The more important biochemical reactions of the Flexner group are summarized in tables 4 and 5. All of its members produce acid without gas from glucose and mannitol. Differences in biochemical activity of various strains with respect to the fermentation of maltose and sucrose were used in the past

for the subdivision of the Flexner group into the following varieties (a) Flexner variety, (b) Hiss-Y-Russell variety, (c) Strong variety (table 5) These fermentation reactions, however, are neither constant in all instances, nor does the resulting subdivision parallel the more important classification based upon the antigenic structure Unfortunately, the biochemical classification of the Flexner group is still largely used in the clinical literature, it should be abandoned

To the medical bacteriologist the differentiation of the Flexner group from other members of the genus *Shigella* is of great importance, particularly, because the epidemiological significance of this group differs greatly from that of closely related species As may be seen from table 4, indole formation and action upon rhamnose, xylose, and dulcitol allow a preliminary differentiation of the Flexner group from other lactose-negative, mannitol-positive members of the genus *Shigella* *S. paradysenteriae* differs from *S. alkalescens* by its inability to produce acid from rhamnose, xylose, and dulcitol It must be mentioned, however, that a few strains of the Flexner group produce acid from rhamnose after incubation for several days (144, and others) It has also been reported that an occasional strain of the Flexner group may ferment dulcitol on prolonged incubation As will be mentioned below, members of the Flexner group can readily be differentiated from *S. alkalescens* by serological methods

*S. paradysenteriae* is comprised of antigenically different types Investigators in different countries have studied the antigenic structure of various strains and proposed different classifications,—a fact which adds more difficulties to an already difficult subject Table 6 presents the various antigenic types of *S. paradysenteriae*, as designated by different authors

Andrewes and Inman (8) have subdivided the Flexner group into five different types, referred to as *S. paradysenteriae* V, W, X, Y, and Z These authors believe that the various members of the group contain four different antigenic components (V, W, X, and Z) and that the respective type is determined by the predominance of one or another of these antigens The presence of minor and varying amounts of the other antigenic components explains the well-known cross-reactions occurring between the different types In *S. paradysenteriae* Y the four antigenic components V, W, X, and Z are rather evenly distributed, it remains doubtful, however, whether this type also contains a fifth antigenic component (Y)

There can be no doubt that the Flexner group is comprised of more than the five original types of Andrewes and Inman (table 6) Sartorius and Reploh (161) have described four additional types, three of which correspond to types reported by Aoki and Murakami Their data do not completely correspond to those of Lentz and Prigge (109) (see tables 2 and 6) The most significant contribution to this subject is that of Boyd (24 to 27), who made an extensive study of the antigenic structure of some four thousand strains belonging to the mannitol-fermenting group of dysentery bacilli About 75% of *S. paradysenteriae* strains are classified as belonging to the five Types V, W, X, Y, and Z of Andrewes and Inman The remaining 25% of his strains are comprised of

about nine additional types. A study of variants of these organisms has led Boyd to a new conception of the antigenic structure of the members of this species, which differs essentially from that of Andrewes and Inman. Boyd gives experimental evidence that the members of the Flexner group may contain a group-specific (species-specific) antigen in addition to type-specific antigens. The group-specific antigen itself is comprised of about six components, some of which are common to various types of the Flexner group. On the other hand, the type-specific antigen characterizes the respective type only. Thus, according to Boyd, the cross-reactions encountered with different types of the Flexner group, are due to common group-specific antigens. At the present time, 13 (possibly 15) different types of *S. paradyserteriae* can be recog-

TABLE 6

*Classifications of the antigenic types of Shigella paradyserteriae according to different authors*

BOYD	ANDREWES AND INMAN	KRUSE	SARTORIUS AND REPLOH	AOKI AND MURAKAMI	SUGGESTED TYPE DESIGNATION
V	V	BC	BC	V	1
W	W	D	D	I	2
X	X	—	X	X	3
Z	Z	H	H	II	4
88	—	—	K(L?)	IX (?)	5
103	—	—	—	—	6
P119	—	—	—	—	7
	Y	—	Y	—	
	VZ	A	A	—	
170	—	—	—	—	8
P288	—	—	—	—	9
P274	—	—	—	—	10
D1	—	—	—	—	11
D19	—	—	—	—	12
P143	—	—	—	—	13
?	—	—	F	III/VI	14 (?)
?	—	—	G	IV/XII	15 (?)
?	—	—	L(K?)	— (IX?)	16 (?)

nized (table 6). They include the original Types V, W, X, and Z of Andrewes and Inman and Types 88, 103, and P119 of Boyd. These seven types contain both group-specific and type-specific antigens. The remaining six types (170, P288, P274, D1, D19, and P143) contain type-specific antigens only. There cannot be any doubt that additional types exist and that some of the types described by Boyd have been studied also by other investigators. Boyd's Type 88 is identical with either Type K or Type L of Sartorius and Reploh, clarification on this point is needed. Whether Types F and G of Sartorius and Reploh are identical with any of the types described by Boyd, remains to be seen. The antigenic relationship of the recently described types to other species of the genus *Shigella* and to other genera needs further investigation.

It is evident that the antigenic heterogeneity of the species *S. paradysenteriae* renders a chemical analysis of the antigenic components particularly difficult.

Mention may be made that the differentiation of various antigenic types of the Flexner group is aided by determination of the susceptibility of the strains under investigation to different pure strains of bacteriophage (34).

Dissociation accompanied by changes in the antigenic structure is rather frequently encountered in members of the Flexner group. These variants may differ from the parent colony in the properties of (a) agglutination, (b) agglutinin-absorption, (c) agglutininogenesis (117, 118). It may be mentioned in passing that, in contradistinction to Shiga's bacillus, members of the Flexner group lack the heterophile sheep-cell hapten, according to Jungeblut and Ross (97). Certain strains of *S. paradysenteriae* share antigens with members of the genus *Salmonella* (Bornstein, Saphra, and Daniels (23)). This latter observation may serve as one of many illustrations of the antigenic interrelationships between different genera and shows that classification of microorganisms should not be based solely on antigenic pattern.

With respect to the pathogenicity of the different types of the Flexner group, it is of interest to note that all of the five Types V, W, X, Y, and Z of Andrewes and Inman may be associated with bacillary dysentery of man (39, 72). Also, some of the new types described by Boyd in 1932 and 1938 (25 to 27) undoubtedly may cause bacillary dysentery, these strains having been isolated (often in almost pure culture) almost exclusively from patients with dysentery. The fact that these patients developed specific agglutinins gives additional evidence of the pathogenicity of the new types of Flexner dysentery bacilli. It is of interest to determine the incidence in other parts of the world of the types of *S. paradysenteriae* described by Boyd.

The question as to whether or not a link exists between the mortality rate and the type of the Flexner group involved deserves further study. Haessler (82) reported a mortality rate of about 30% in his 24 cases of infections due to *S. paradysenteriae*, Type D (cf. table 6), while the mortality rate was considerably lower in infections due to other types. In our series of more than 20 cases of dysentery caused by Type D no death occurred (Neter, unpublished data). Teveli and Tamasi (177) noted no differences in the severity of infections due to different types.

The Widal test for the diagnosis of dysentery due to members of the Flexner group is rendered very difficult because of their antigenic complexity. In the first place, it necessitates the use of several antigenic types. In the second place, no hard and fast rules can be given with respect to agglutinin titers indicative of infection. The titer of normal agglutinins of human sera for members of the Flexner group may range from 1:10 or less up to 1:400 and above, even when identical experimental conditions are observed. Repeated examinations give more reliable results than a single determination of the agglutinin titer. It may be mentioned that sera of patients with infections due to Shiga's bacillus may also agglutinate certain of the Flexner dysentery bacilli. It is regrettable that the Widal test for the diagnosis of infections with

Flexner bacilli has been used quite frequently without due consideration of the points just outlined, and that conclusions regarding the etiology of chronic ulcerative colitis have been based on such results

*S. paradysenteriae* is markedly less toxic for experimental animals than *S. dysenteriae*. In contrast to the latter organism, Flexner dysentery bacilli do not produce a powerful exotoxin. On the other hand, living or even killed suspensions of *S. paradysenteriae* may cause lesions and death of animals, attributable to the action of endotoxins present within the bacterial cells. The fact that Flexner dysentery bacilli do not produce an exotoxin may account for the clinical observation that dysentery of man due to these organisms usually appears to be less toxic than dysentery caused by Shiga's bacillus.

8 *S. alkalescens* (Andrewes) Weldin was first described in 1918 by Andrewes (7). It resembles *S. paradysenteriae* in many respects and has been quite frequently confused with it. As a matter of fact, a strain of *S. alkalescens* has been used for the production of a commercially available "Flexner antiserum"! *S. alkalescens* is a non-motile, gram-negative bacillus that grows well on ordinary culture media, including Endo agar. On agar the colonies are usually of the S form, occasionally, R variants may be seen. The biochemical reactions of this organism are very characteristic, the more important ones are summarized in table 4. Like *S. paradysenteriae*, *S. alkalescens* produces acid without gas from glucose, maltose, and mannitol. In litmus milk, following transitory acidification, it produces a characteristic strong alkaline reaction. Indole is always produced. As mentioned above, it differs from Flexner's bacillus in its capacity to produce acid, usually within 24 to 48 hours, from dulcitol, rhamnose, and xylose. Furthermore, it may be differentiated from *S. paradysenteriae* by means of the acid-agglutination test, according to Andrewes (7). *S. alkalescens* is agglutinated in test solutions of pH 2.2 to 3.2, whereas *S. paradysenteriae* is not clumped under identical conditions. These findings, however, have not been completely confirmed by other authors. The possibility has to be considered that the susceptibility to acid-agglutination of S and R forms of Flexner's bacillus may not be the same. From a practical point of view, acid-agglutination is superfluous, because serological methods allow a much more reliable differentiation between these two species.

Antigenically, *S. alkalescens* has been considered until recently to form a homogeneous species (7, 189, 139, 145). Recently, however, Assis (13) has described two different antigenic types (Types I and II). Only a few Type II strains have been described thus far (13). Of more than 40 strains of *S. alkalescens* isolated in this laboratory all were of Type I.

*S. alkalescens* (Type I) is antigenically related to *S. paradysenteriae* antisera to *S. paradysenteriae* may agglutinate to high titer *S. alkalescens* and, vice versa, antisera to *S. alkalescens* may agglutinate certain types of Flexner dysentery bacilli. However, it has been shown that *S. alkalescens* (Type I) contains a species-specific antigen which is not shared by Flexner dysentery bacilli. Absorption of an antiserum to *S. alkalescens* with a suspension of Flexner dysen-

tery bacilli (Types V, W, X, Y, and Z) eliminates all antibodies to the Flexner bacilli without reducing the titer of agglutinins to *S. alkalescens* (140)

*S. alkalescens* may be found in the intestinal tract of man free of enteric disease (Snyder (172)) Convincing evidence is accumulating, however, that this organism may cause mild and even severe forms of dysentery or enteritis (12, 13, 64, 133) It also may cause other diseases such as septicemia and infections of the urinary tract (170, 173, 194, 137) Further evidence of its potential pathogenicity is the observation that patients infected with this microorganism may develop specific agglutinins during the course of the disease (139)

9 *S. gallinarum* (Klein) Weldin is a non-motile bacillus Its more important biochemical characters are shown in table 4 It produces acid from glucose, mannitol, maltose, rhamnose, xylose, and dulcitol, but fails to attack lactose and sucrose Indole is not formed, and gelatin is not liquefied It produces  $H_2S$  The reports on its capacity to reduce nitrates and to cause changes of the pH in litmus milk are conflicting (99, 123)

It is interesting to note that a similar organism, *S. gallinarum* (var *Duisburg*), has been described recently by Müller (131) as the cause of acute gastro-enteritis in man This microorganism was studied by Kauffmann (98) It differs from *S. gallinarum* in its inability to form  $H_2S$ , its slow production of acid from maltose, and its failure to form acid from d-tartrate

Antigenically, *S. gallinarum*, *S. gallinarum* (var *Duisburg*), and *Salmonella pullorum* are identical, inasmuch as they contain the O-antigen of Group D of the genus *Salmonella* It is interesting to point out that this particular somatic antigen is shared also by *E. typhosa* Thus, species now considered to belong to three different genera contain a common antigen Some bacteriologists consider *S. gallinarum* as a member of the genus *Salmonella*, more specifically as a variety of *S. pullorum*

It may be mentioned that *S. gallinarum* differs in its antigenic structure from *S. pfaffii*

St John-Brooks and Rhodes (175) have studied *S. gallinarum* in detail and compared it with *S. jeffersonii* of Hadley and his associates S colonies of the latter were found to be identical with those of *S. gallinarum* in every respect, including antigenic structure Apparent differences between these two organisms were those of R and S dissociation Thus, there is no longer any reason to consider *S. jeffersonii* as a distinct species of the genus *Shigella*

10 *S. pfaffii* (Hadley, et al ) Weldin was first isolated from canaries suffering from septicemia Its distribution and its pathogenicity for animals other than the canary remain to be determined It is unknown whether this microorganism, like *S. gallinarum*, may cause gastro-enteritis or other diseases in man In Bergey's Manual of Determinative Bacteriology (19) and in Kelser's Manual of Veterinary Bacteriology (99) it is described as non-motile According to St John-Brooks and Rhodes (175), however, it is considered to be motile

If the latter observation is correct, this organism should be eliminated from the genus *Shigella*

*S. pfaffii* produces acid without gas from glucose, mannitol, maltose, rhamnose, and xylose, but not from lactose, sucrose, and dulcitol (table 4). It does not produce indole, does not reduce nitrates, and fails to liquefy gelatin. Its inability to ferment dulcitol and its antigenic structure differentiate it distinctly from *S. gallinarum* (175). A thorough reinvestigation of this species is definitely needed.

11 *S. rettgeri* (Hadley, *et al.*) Weldin was first isolated in 1909 by Rettger from a cholera-like epidemic in chickens. Its distribution and its pathogenicity for various animals and man are not known.

*S. rettgeri* is a non-motile bacillus,<sup>3</sup> it produces acid without gas from glucose, mannitol, and xylose, but does not attack lactose, sucrose, maltose, and dulcitol. Litmus milk is rendered alkaline (table 4). According to Hadley and to Bergey's Manual (19), this organism does not produce indole, St John-Brooks and Rhodes (175), however, obtained positive results. *S. rettgeri* does not liquefy gelatin. A thorough reinvestigation of this microorganism is desirable, particularly in regard to indole production, incidence, and pathogenic significance.

#### GROUP III THE LACTOSE-POSITIVE, MANNITOL-NEGATIVE MEMBERS

Any attempt to discuss the lactose-positive, mannitol-negative members of the genus *Shigella* is confronted with great difficulties. In the first place, organisms of this group (with the exception of *S. gintottensis*) have not been studied adequately and have received but scant attention during recent years. In the second place, the data available in various textbooks, manuals, and dictionaries do not correspond. It appears likely that some of the organisms do not belong to this group or even to the genus *Shigella*. A thorough reinvestigation of the available strains seems necessary. Otherwise, our knowledge of these species will remain as it was several decades ago.

12 *S. gintottensis* (Castellani) Hauduroy, *et al.* was first described by Castellani many years ago. Since then, little has been added to our knowledge of this species. This organism has been recovered from the feces of patients with dysentery and has been considered as the cause of this disease. According to Castellani (37), it is a non-motile, gram-negative bacillus, it produces acid from glucose, and may or may not ferment lactose, it does not attack sucrose,

<sup>3</sup> According to Dr. P. R. Edwards, Department of Animal Pathology, University of Kentucky, Lexington, Kentucky (personal communication), a supposed descendant of the original strain of Hadley's culture spreads readily in semi-solid agar when transfers are made from the projecting growth. Continued transfer in this medium gives rise to a very actively motile culture which possesses the cultural and biochemical properties of the original strain. Thus, it appears that *S. rettgeri*, like *S. pfaffii*, should be eliminated from the genus *Shigella*.

mannitol, maltose, and dulcitol, it causes acid and clot formation in litmus milk, it does not form indole (table 7). If certain strains of this species do not produce acid from lactose, as stated by Castellani (37), it is hardly justifiable to include them in this particular group of the genus *Shigella*. Certainly, it should not be classified among the mannitol-fermenting species, as it has been in Bergey's Manual (19).

13 *S. birsteinii* (Schroeter) Bergey, *et al* is a non-motile, gram-negative bacillus. According to the description in Bergey's Manual of Determinative Bacteriology (1934) (18), it produces acid from glucose and lactose. Ford (66), however, states that it fails to ferment lactose and sucrose and produces

TABLE 7

Biochemical reactions and antigenic structure of the lactose-positive, mannitol-negative group\*  
(Data for xylose not available)

	INDOLE	LITMUS MILK	GLUCOSE	LACTOSE	SUCROSE	MANNITOL	MALTOSE	DULCITOL	ANTIGENIC STRUCTURE
<i>Shigella gintottensis</i> (Castellani), Hauduroy, <i>et al</i> †	—	AC	A	A or —	—	—	—	—	Unknown
<i>Shigella birsteinii</i> (Schroeter), Bergey, <i>et al</i>	—	AC	A Alk ‡	A — ‡	— ‡	—			Unknown
<i>Shigella oxygenes</i> (Ford), Bergey, <i>et al</i>	—	AC	A Alk ‡	A — ‡	— ‡				Unknown

A = Acid reaction      Alk = Alkaline reaction

C = Coagulation      — = No indole or acid production

\* Compiled from Bergey's Manual of Determinative Bacteriology (18)

† According to Castellani (36)

‡ According to Ford (66)

an alkaline reaction in glucose broth. It does not form indole. It causes acid and clot formation in litmus milk. Gelatin is not liquefied (table 7).

14 *S. oxygenes* (Ford) Bergey, *et al* was first isolated by Ford in 1901. It is a non-motile, gram-negative bacillus. According to Ford (66), it does not ferment sucrose and lactose and produces an alkaline reaction in glucose broth. In Bergey's Manual of Determinative Bacteriology (1934) (18), it is described as an organism which produces acid in glucose and lactose. It causes acid and clot formation in litmus milk. It does not form indole and fails to liquefy gelatin.

#### GROUP IV THE LACTOSE-POSITIVE, MANNITOL-POSITIVE MEMBERS

15 *S. sonnei* (Levine) Weldin. Late-lactose-fermenting members of the genus *Shigella* were described as early as 1904 by Duval (54, 55) and 1907 by

Kruse (105), and since then by Castellani (36 to 38). However, it was not until 1915, that Sonne (174) proved beyond doubt that such an organism may cause dysentery in man. His conclusions were based on a thorough investigation and his findings may be summed up as follows: (a) Sonne isolated this particular species from patients with bacillary dysentery, in some instances in almost pure culture. (b) These patients developed agglutinins toward this bacillus in titers ranging from 1:25 to 1:250, whereas sera of normal persons very rarely agglutinated this organism in titers of 1:10 or above. (c) With the exception of one carrier, this microorganism was not found in the intestinal canal of normal individuals. (d) Finally, Sonne succeeded in producing a dysentery-like disease in a monkey by feeding one of his strains. Today, this bacillus is referred to as *S. sonnei*, sometimes also as the Duval-bacillus, Duval-Sonne bacillus, Kruse-Sonne bacillus, Kruse-E-bacillus, and *B. ceylonensis* A (Castellani).

The Sonne dysentery bacillus grows well on ordinary media, often somewhat more luxuriantly than either Shiga or Flexner dysentery bacilli. The cultural characters of a large number of strains have been thoroughly investigated by Dienst (48), Koser and Dienst (101), Chinn (40) and Glynn and Starkey (74). Upon isolation on lactose-containing culture media, Sonne's bacillus first appears as a non-lactose-fermenting colony. After incubation for 24 hours on agar the colonies are rather flat, somewhat granular and opaque and have a diameter of 3 to 4 mm. The colonies present a smooth, raised, central zone grading out to a thin slightly irregular edge. Upon further incubation the size of the colony increases. Papillae or daughter colonies appear, which consist of raised, smooth, entire, rounded outgrowths on the surface. Forty-eight hours after their appearance these daughter colonies are usually 1 to 2 mm in diameter. Characteristically, they ferment lactose. A few strains fail to develop papillae even upon incubation for two months. Hall (84) as well as Sears and Schoolnik (165) showed that the late fermentation of lactose is due not to a slow utilization of this carbohydrate, but to the appearance of lactose-fermenting daughter colonies. Essentially the same holds true for the action on sucrose (154). Many different colony forms can be obtained from older cultures on agar. Among them are flat, entire colonies with bevelled edges and a moist shiny surface. According to Glynn and Starkey (74), this particular colony form does not breed true and does not give rise to daughter colonies.

Of great interest are the G or G-like colonies of *S. sonnei*, which were investigated by Dienst (48), Koser and Dienst (101), and Chinn (40). These colonies on agar have a diameter of 0.012 to 0.2 mm as compared to a diameter of 1.5 to 2.5 mm of normal sized colonies. It is worth emphasizing that G or G-like colonies of *S. sonnei* are obtained infrequently and irregularly. Chinn (40) found dwarf colonies to represent only about 1% of all colonies of old strains. Biochemically, the organisms of G colonies are markedly less active than those of the parent colonies. This may be due to (a) the relatively slow growth of the organism and (b) absence or low concentration of certain constitutive enzymes or inability of the organisms to form adaptive enzymes. The

fact that growth-promoting substances such as serum increase the biochemical activity of the G type colony, is in favor of the first possibility. On the other hand, it should be pointed out that even in the presence of serum some G type colonies fail to produce acid from sugars which are fermented by normal sized colonies. Antigenically, certain G type colonies seem to be related to normal strains. Some, but not all G type colonies of *S. sonnei* revert to normal sized colonies upon prolonged incubation on agar or following repeated serial transfers to broth or agar. Thus far, G colonies of *S. sonnei* have not been recovered directly from lesions in man. In this connection it is important to point out that G colonies of *S. equirulis* have been isolated directly from foals. There is no convincing evidence for the existence of filtrable forms of *S. sonnei*. Only one of the strains of Chinn (40) passed through N filters, this strain, however,

TABLE 8

*Biochemical reactions and antigenic structure of the lactose-positive, mannitol-positive group*

	INDOLE	LITMUS MILK	GLUCOSE	LACTOSE	SUCROSE	MANNITOL	MALTOSE	RAMNOSE	XYLOSE	DULCITOL	ANTIGENIC STRUCTURE
<i>Shigella sonnei</i> (Levine) Weldon	—	A-AC	A 24 hrs	A 1-20 days	A 1-20 days	A 24 hrs	A 24 hrs	A 24 hrs	—	—	2 types
<i>Shigella equirulis</i> (de Bleeck and van Heelsbergen), Edwards	—	A	A	A	A	A	A	—	A	—	Heterogeneous
<i>Shigella ceylonensis</i> B (Castellani), Weldon	+	AC	A 3 days	A 3-14 days	A 3-7 days	A 3 days	A 3 days	A 3 days	A 3 days	Slight A 3 days or — 14 days	Homogeneous (?)
<i>Shigella madampensis</i> (Castellani), Weldon	+	AC	A 3 days	A 3-14 days	A 3-7 days	A 3 days	A 3 days	A 3 days	A 3 days	—	Probably heterogeneous

A = Acid reaction      + = Indole production  
C = Coagulation.      — = No indole or acid production

did not revert to normal. All other experiments have failed to reveal filtrable forms of this organism.

The biochemical characters of the Sonne dysentery bacillus are summarized in table 8. From a practical diagnostic standpoint it is noteworthy that the fermentation of lactose requires several days and sometimes a few weeks. The differentiation of Sonne's from Flexner's bacillus is aided by testing the capacity to ferment rhamnose. The Sonne bacillus usually produces acid from rhamnose in broth within 24 to 48 hours, whereas most strains of Flexner dysentery bacilli fail to do so or produce acid only after incubation for several days, as shown by Hilgers (92) and confirmed in this laboratory. The inability of the Sonne bacillus to produce indole differentiates this organism distinctly from *S. dispar*, *S. ceylonensis* B and *S. madampensis* (table 8).

Until recently, the Sonne bacillus was considered to represent an antigenically homogeneous species (103, 94). Recent investigations by Glynn and Starkey

(74), however, have revealed two immunological types. Type I contains one of two antigens in predominance, whereas Type II contains both of these antigens in considerable amounts. This conclusion is based upon the following observations: antiserum to Type II agglutinates to full titer both Types I and II strains, whereas antiserum to Type I agglutinates Type II organisms only to a fraction of its titer. Likewise, Type II organisms completely absorb the agglutinins from antisera to both Types I and II, while Type I organisms absorb not more than 50 to 75 per cent of the agglutinins from a Type II serum. These findings are not only of theoretical interest, but also of practical significance. It follows that a serum containing antibodies directed against Type II will agglutinate all agglutinable strains and, therefore, is the serum of choice for diagnostic purposes. In this connection it may be mentioned that some freshly isolated strains of Sonne bacilli are not agglutinated by the homologous antiserum, but may absorb the respective antibodies (69). The absorption experiment, therefore, may be successfully employed when inagglutinable strains are encountered. Attention may be called to the fact that some so-called inagglutinable strains may be agglutinated by applying certain procedures, e.g., prolonged incubation, incubation at higher temperatures (45 C to 55 C instead of 37 C), and by centrifugation of the serum-suspension mixture. Zeithaml and Ecker (196) obtained specific agglutination by centrifugation of Sonne's dysentery bacilli which were otherwise not agglutinated.

The Sonne bacillus shares minor antigenic components with some other members of the genus *Shigella*, namely, *S. paradysenteriae* (74). The cross-reactions between these organisms, however, are not very marked and generally do not interfere with diagnostic agglutination tests.

16 *S. equirulis* (de Bleeck and van Heelsbergen) Edwards is the causative agent of a disease of young foals that is characterized by arthritis, nephritis, and septicemia. The organism was most thoroughly studied by Edwards (56). Morphologically, it is highly pleomorphic. It is interesting to note that on agar very young cultures (8 to 10 hours old) frequently show long, filamentous forms and streptococcus-like chains, as well as large yeast-like bodies bearing projections. The microscopic appearance of the organisms of older cultures (14 to 16 hours) parallels to a certain extent the characters of the colony. Rough, mucoid colonies consist almost entirely of short oval rods, whereas smooth colonies contain many long filamentous forms and streptococcus-like chains. Edwards made the important observation that rough colonies of *S. equirulis* are always mucoid and that non-mucoid colonies are always smooth. The trend of the variation in artificial cultures is from rough to smooth. Smooth mucoid colonies are found as a transitional stage.

R forms of *S. equirulis* are very mucoid, they have a dry, dull surface, the edges are undulate and the surface is extremely rough. In broth growth appears first on the side of the culture tube, later, a light pellicle is formed on the surface, and the sides of the tube are covered with masses of bacteria. Still later, a diffuse growth may occur. Smooth colonies on agar, on the other hand,

are flat or slightly raised, non-mucoid, and have a perfectly smooth surface, in broth an even clouding occurs. It must be noted, however, that the relation of the features of cell morphology and mucoid nature of the rough phase, as reported by Edwards, differs from findings in other species and that such an expert on problems of bacterial dissociation as Hadley (79) raises the question as to whether the mucoid-rough cultures of Edwards may have been impure. Nevertheless, the observation of Edwards of the existence of a mucoid phase in a member of the genus *Shigella* itself is of great importance.

In addition to rough and smooth colonies of *S. equirulis*, dwarf colony variants may occur. These dwarf colonies are composed of short oval rods. On agar they appear after an incubation period of 4 days or longer and usually have the same characters in regard to rough or smooth consistency as the parent colonies (56). In contrast to normal strains, which grow readily in extract broth, these dwarf variants do not grow at all or only very poorly in this culture medium.

Biochemically, *S. equirulis* forms acid from glucose, levulose, galactose, maltose, lactose, sucrose, xylose, raffinose, and mannitol, no acid is produced from rhamnose, dulcitol, and sorbitol. Indole is not formed and gelatin is not liquefied. It reduces nitrates to nitrites. Litmus milk is rendered acid and some strains cause coagulation of the milk and reduction of the litmus. As may be seen from table 8, *S. equirulis* may be readily distinguished from the Sonne dysentery bacillus by its inability to ferment rhamnose and its capacity to ferment xylose.

Antigenically, *S. equirulis* is a heterogeneous species. Both smooth and rough colonies of a single strain possess a common antigen. It is interesting to note that, as shown by Edwards (56), dwarf colonies are antigenically related to the normal strains.

Edwards (56) has made an observation of great general interest, namely, that both rough and smooth forms as well as dwarf colonies of *S. equirulis* can be isolated from the tissues of infected foals, indicating that all three forms of this microorganism may be pathogenic.

It has been suggested that this species might be a member of the genus *Actinobacillus* (unpublished suggestion, cf. Bergey's Manual (19)).

17 *S. ceylonensis* B (Castellani) Weldon. 18 *S. madampensis* (Castellani) Weldon. *S. ceylonensis* B and *S. madampensis* were originally described by Castellani in 1907 and 1911, respectively, the former microorganism was isolated from the feces of patients with the clinical signs of dysentery, and the latter from patients with colitis and cystitis. In 1918, Andrewes (7) described certain lactose-fermenting microorganisms under the name of *Bacillus dispar*. Subsequent investigations revealed that strains described as *Bacillus dispar* are identical with either *S. ceylonensis* B or *S. madampensis*. Thus, at the present time, *S. dispar* can not be recognized as a distinct species. *S. ceylonensis* B and *S. madampensis* show only minor differences. It seems reasonable to suggest, therefore, that the organisms under consideration be classified as a

single species (*S. castellanii*), just as *S. paradysenteriae* comprises organisms with certain biochemical and antigenic differences

Colonies on agar of strains classified as *S. dispar* appear very similar to colonies of the Sonne bacillus. They, too, may show secondary papillae. The biochemical characters of *S. ceylonensis B* and *S. madampensis* are almost identical, as may be seen from table 8. *S. ceylonensis B* differs from *S. madampensis* in its ability to produce acid from dulcitol. Indole production distinctly differentiates *S. ceylonensis B* and *S. madampensis* from the Sonne bacillus. Furthermore, according to Forsyth (68), strains identified as *S. dispar* are methyl-red-positive, whereas *S. sonnei* is not.

The antigenic structure of these organisms is not as yet fully elucidated. Castellani (36 to 38) reported that both *S. madampensis* and *S. ceylonensis B* are antigenically homogeneous species. However, it should be noted that according to Glynn and Starkey (74) strains labeled as *S. dispar*, which were identical with strains of *S. madampensis*, proved to be antigenically heterogeneous. Furthermore, Forsyth (68) found that strains identified as *S. dispar* form an antigenically heterogeneous species. Further studies are needed before final conclusions can be drawn in regard to the antigenic pattern of the species under consideration. Then, it will be possible also to determine the antigenic relationship of these organisms to other species of the genus. Minor antigenic relationships between *S. dispar* and Sonne and Flexner dysentery bacilli were found by Watanabe (186) and by Welch and Mickle (188).

No conclusive evidence is available at the present time that these organisms are a cause of epidemic or endemic dysentery of man. Members of this group of organisms may be found in the feces of healthy individuals and of patients with or without intestinal diseases. Johnston and Kaake (95) reported cases of enteritis in children from whom *S. dispar* was isolated. It remains to be seen whether these organisms are the primary incitants of the disease or only secondary invaders. Occasionally, these organisms may cause infections of the urinary tract and purulent lesions originating from the intestinal canal.

#### GENERAL CONSIDERATIONS

Many species of the genus *Shigella* have been studied thoroughly in the past and much information of general bacteriological and immunological interest has been brought to light. However, it is evident from a review of the available reports that other species, now classified as members of this genus, have not been adequately investigated, and little is known in regard to their cultural characters, biochemical activities, and antigenic structure. It is safe to assume that some of these microorganisms may not even belong to the genus. It seems highly desirable that competent investigators collect and study as many strains as possible of these little known species such as *S. septicaemiae*, *S. minutissima*, *S. pfaffii*, *S. rettgeri*, and others. Only then, will it be possible to reconsider their taxonomic position, to identify with accuracy strains isolated from various sources, and to determine their significance in diseases of man and animals.

Until recently, our knowledge of the specific growth requirements of the members of the genus has been inadequate. Investigations similar to those of Koser and his associates, when they are extended to include all the members of the genus, may yield interesting information and aid greatly in the classification of these microorganisms.

One of the most significant recent contributions to our knowledge of the genus is that of Boyd. His demonstration of a group-specific antigen in certain types of *S. paradysenteriae* may form a basis for similar investigations of other species and genera. The observations of Boyd are also of practical importance and should be considered in any attempt to produce either active or passive immunity to infections with the Flexner group of dysentery bacilli. The chemical analysis of antigenic components of some species of the genus has yielded interesting information during the last few years. Such investigations have been particularly fruitful at the hands of Morgan and his associates. It is to be hoped that their studies on Shiga's dysentery bacillus will be extended to other species.

One of the least understood problems connected with the genus *Shigella* in particular is that of pathogenicity. At the present time, with the exception of the Shiga exotoxin, very little is known in regard to the factors in dysentery bacilli responsible for dysentery of man, and it has not been explained why one species may cause epidemic outbreaks of this disease and a very closely related species does not. Future investigations may shed light on these problems.

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# THE PATHOGENIC RICKETTSIAE WITH PARTICULAR REFERENCE TO THEIR NATURE, BIOLOGIC PROPERTIES, AND CLASSIFICATION

HENRY PINKERTON

Department of Pathology, Saint Louis University School of Medicine, St. Louis, Missouri

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The growth of our knowledge of the pathogenic rickettsiae in recent years forms one of the most fascinating chapters in the field of microbiology. Intensive study of these organisms has paved the way for the control of the important human diseases which they produce. Of still greater importance, perhaps, is the light which these studies have thrown on problems of intracellular parasitism in general.

Historically it is of interest that, following early unsuccessful attempts to solve the etiology of typhus and Rocky Mountain spotted fever by simple bacteriological methods, these infections were thought to be caused by viruses. Subsequently, minute intracellular organisms, morphologically similar to bacteria, were associated with these diseases. These organisms were first found in the tissues of the arthropod vectors. They were called rickettsiae, for reasons which will be brought out below, and were considered to belong to a new group of pathogenic agents, distinct from bacteria and viruses. Recent developments indicate that the rickettsiae have many features in common with certain bacteria and with some of the viruses and suggest that the attempt to separate these three groups of pathogens sharply from one another may be unwise. In order that the rickettsiae may be considered in proper perspective, a brief discussion of their inter-relationship with bacteria and viruses will be given.

## BIOLOGIC RELATIONSHIP TO OTHER PATHOGENS

Among the pathogenic organisms at present classed as bacteria, there is great variation in the ability to carry out independent metabolic activities (1). Certain organisms, like the colon bacillus, live and multiply freely in media containing simple carbon compounds, ammonia, and inorganic salts. These bacteria possess enzyme systems of a complex nature, by virtue of which they are able to carry out the chemical transformations necessary for their free life and

reproduction Other bacteria, like the influenza bacillus, have a less complete enzymatic equipment, and therefore require more complex media containing organic substances which are found only in animal tissues or in extracts of animal tissues

Organisms of the group last mentioned are, therefore, somewhat dependent for their food supply on living cells of the animal which they parasitize Like those of the first group, however, they grow in the intercellular fluids of the infected animal and are practically never seen within cells, except insofar as they may be engulfed by the defensive phagocytic cells of the body The cultivation of these organisms on bacteriological media, though somewhat more difficult than in the case of the colon bacillus, is accomplished with relative ease, because the compounds on which they live can be obtained in a fairly stable form by extraction from living tissues

A still greater degree of dependence on living animal tissues is shown by organisms like *Pasteurella tularensis* and *Bartonella bacilliformis* These organisms, in infected tissues, multiply extra-cellularly under certain conditions, but under other conditions show a decided preference for the interior of cells, multiplying freely in the cytoplasm Their growth in bacteriological media is achieved only by the careful reproduction of certain necessary chemical and physical conditions The beneficial effect of cystine in the cultivation of *P. tularensis*, for example, is of great interest because of the fact that this substance is of importance in intracellular oxidation-reduction processes It is probable that in adding cystine to our artificial medium we are supplying a metabolic activator for the action of which the organism, in nature, depends on living cells of the animal which it infects Similarly, German (2) has recently reported that the addition of ascorbic acid and glutathione to culture media facilitates the growth of *Bartonella bacilliformis*

The next stage in the biological adaptation to growth in living cells is represented by the obligate intracellular parasites Conditions suitable for the multiplication of these pathogens are found only within the cytoplasm or nucleoplasm of living or surviving cells This group includes the typical pathogenic rickettsiae and probably all of the viruses, although direct proof that certain invisible viruses multiply only within cells has perhaps not been furnished In tissues infected with pathogenic rickettsiae, we find colony-like masses of organisms in the cytoplasm or nucleus of cells Similar intracellular colony-like masses of elementary bodies are associated with the presence in tissues of certain of the larger viruses, such as psittacosis (32) (intracytoplasmic) and herpes (89) (intranuclear) In tissues infected with the smaller viruses, we often find spherical intracellular bodies (the so-called inclusion bodies) which appear homogeneous and which may or may not be agglomerated masses of elementary bodies, with or without the addition of other elements derived from the host cells Limitations of microscopy have thus far made it impossible to determine the exact nature of many of these inclusions, but it seems possible that some of them may not differ essentially from colonies of rickettsiae or elementary bodies The nature of the inclusion bodies associated with the

smallest viruses, which approach molecular size, remains a matter of speculation (3)

Present evidence indicates that the rickettsiae, although they are as strictly dependent on intracellular conditions as the smaller viruses, have a somewhat more complex enzyme system than the latter, and are therefore able to maintain a certain amount of independent metabolic activity within their host cells. Thus we may consider the rickettsiae, both from the point of view of size and from the point of view of independent metabolic activity (enzymatic complexity) as occupying a position intermediate between certain cytotropic bacteria and the viruses

#### TAXONOMIC CONSIDERATIONS

*Historical* In dealing with a new group of infective agents, it is inevitable that early attempts at nomenclature and classification, based upon incomplete knowledge of their biological properties, should lead to considerable confusion. Even at the present time, criteria suitable for a satisfactory classification are not available, and any attempt to classify the rickettsiae must be considered as having only a tentative and pragmatic value. In order to understand the taxonomic difficulties which obtain at present, a brief discussion of nomenclature from the historical aspect seems desirable.

The term "Rickettsia" was applied in 1916 by da Rocha-Lima (4) to certain minute microorganisms found in the intestinal tract of lice. The name honors the memory of Howard Taylor Ricketts, who with Wilder first described in 1910 organisms of this type in lice fed on typhus patients (5, 6). da Rocha-Lima showed, by studying sections of lice, that the organism acquired by feeding on typhus patients was an intracellular parasite, distending the epithelial cells of the louse's stomach. He believed this organism to be the etiological agent of typhus, and subsequent work, notably that of Wolbach, Todd, and Palfrey (7) published in 1922, has established the truth of this concept. To this organism, da Rocha-Lima gave the name *Rickettsia prowazeki*, the specific name being in honor of von Prowazek, who, like Ricketts, died of typhus acquired in the course of his pioneer investigations.

Organisms morphologically similar to *R. prowazeki* but extracellular in habitat were found in lice in association with trench fever by Töpfer (8, 9) in 1916, and in presumably normal lice by Munk and da Rocha-Lima (10) in 1917. The latter workers gave to these organisms the name *Rickettsia pediculi*, although they fully realized that they differed from *R. prowazeki* in that they multiplied extracellularly in the digestive tract of the louse. The probable relationship of such extracellular organisms to trench fever will be discussed below.

*Non-pathogenic rickettsiae* During the next decade approximately forty-two microorganisms, some of intracellular and some of extracellular habitat, were described in the tissues of thirty-seven species of arthropods. Information concerning these organisms was in most cases confined to the mere fact that they could be seen in certain locations in the tissues of certain arthropods, and the possibility that the same organism might be present in two or more arthropods

was not excluded. It was natural that the general term rickettsia should be applied to these organisms, since for the most part they resembled *R. prowazeki* rather closely. The generic name *Rickettsia*, together with a specific name, was given to a number of them. In many cases, this was unfortunate. *Rickettsia melophagi* (11), for example, an extracellular organism found in the intestine of the sheep ked, and rather easily cultivated on ordinary bacteriological media, has little in common with the more characteristic rickettsiae, which are obligate intracellular parasites.

This large group of organisms in insect tissues is often referred to as the "non-pathogenic rickettsiae," a term indicating their lack, so far as our knowledge goes, of pathogenicity for mammals. For the most part, they are also non-pathogenic for their insect hosts, and in many instances are transmitted hereditarily from one generation to another by way of infected ova, apparently living in complete harmony with their host cells in the insect tissues.

Closely related to the rickettsiae, and at times not sharply separable from them, are the so-called symbionts, some of which appear to exert a useful or even essential function in the metabolism of insect tissues. For a general discussion of the microorganisms and other structures found in insect tissue, and of their relation to one another, the reader is referred to a recent paper by Steinhaus (12).

Presumably the rickettsia of typhus fever (*R. prowazeki*), and other pathogenic rickettsiae which will be discussed below, belong in the same general group as many of the non-pathogenic rickettsiae described above, and their pathogenicity for mammals is purely accidental or even acquired. Antigenic relationship between pathogenic and nonpathogenic rickettsiae has not, however, been demonstrated (13).

Most of the non-pathogenic rickettsiae have been identified and named entirely on the basis of their morphology and distribution in insect tissues. The limitations of this method of study are obvious. Organisms which look alike may be physiologically very different, and, since pleomorphism may occur in the rickettsiae, morphologically dissimilar organisms may actually be identical. Although many careful and valuable studies of a purely morphological nature have been made, and in some instances the assignment of generic and specific names may have been justified, the accurate classification of the non-pathogenic rickettsiae must in general await the development of methods for isolating them and for determining their biological properties.

*Pathogenic rickettsiae*—The rickettsiae that are pathogenic for mammals may be isolated and identified much more readily than the non-pathogenic rickettsiae. When an organism invades mammalian tissues and produces constantly a characteristic infection, we may be sure that we are dealing with a definite entity. The tissues of ticks may contain non-pathogenic rickettsiae as well as *Dermacentor* *centro* *xenus rickettsi*, the cause of spotted fever, and the two types of organisms may, except for the invasion of nuclei by the latter, be morphologically indistinguishable (13). By feeding such ticks on guinea pigs, however, or by injection of the tick viscera into guinea pigs, we can readily separate the pathogenic organism from the non-pathogenic organisms, since only the pathogenic one

will invade the mammalian tissues. Immunological tests on guinea pigs recovering from rickettsial infection serve to identify the pathogenic rickettsiae with great precision. Attempts to define and classify the pathogenic rickettsiae have for these reasons been relatively successful.

Using as a basis the criteria suggested by Cowdry (14) in 1923, by Wolbach (15) in 1924, and by Cowdry (16) in 1926, the rickettsiae may be characterized as follows: *small, often pleomorphic, gram-negative, bacterium-like organisms, living and multiplying in arthropod tissues, behaving as obligate intracellular parasites, and staining lightly with aniline dyes. With few exceptions, criteria adequate for classification on the basis of biological properties are available only for those members of the group which are pathogenic for mammals.* At present, only four pathogenic organisms can be definitely placed in the group if we define it thus rigidly: *Rickettsia prowazekii*, the cause of louse- and flea-borne diseases of the typhus group, *Demacentrozetes rickettsi*, the cause of tick-borne diseases of the spotted fever group, *Rickettsia tsutsugamuchi* (synonym, *R. orientalis*), the cause of mite-borne diseases of the tsutsugamuchi group, and *Rickettsia ruminantium*, the cause of tick-borne "heartwater" in sheep, goats, and cattle.

Wolbach (17) in 1919 believing that the etiological agent of Rocky Mountain spotted fever showed more than species differences from *Rickettsia prowazekii*, the cause of typhus, gave the former organism a different generic name (*Demacentrozetes*). Subsequent work has substantiated the rather wide differences between the two organisms, and it is to be regretted that organisms more recently added to the group of rickettsiae have not likewise been placed in different genera since, almost without exception, they have shown differences of sufficient magnitude to make such assignment appear desirable.

The author (18) has suggested that the pathogenic rickettsiae, as a tentative working basis, be considered as members of a bacterial family, the RICKETTSIACEAE. (The reasons for not separating them from bacteria will be brought out below.) This plan allows for the creation of new genera, if this seems advisable, for newly discovered organisms which possess the necessary characteristics for inclusion in the group but which show differences from the type genus and species, *Rickettsia prowazekii*, of such importance that they are regarded as of more than specific value. The term rickettsiae, spelled with a small letter, has become firmly established in the literature and may be used loosely as a synonym for the RICKETTSIACEAE, much as we use the term actinomycetes for various species of ACTINOMYCETACEAE.

Although the family RICKETTSIACEAE was created for pathogenic rickettsiae, non-pathogenic organisms, when sufficiently well studied to show that they probably are distinct entities and possess the required characteristics, may tentatively be included. Hertig (19), for example, has described an organism of constant occurrence in the gonads of the mosquito, *Culex pipiens*. To this organism he gave the name *Wolbachia pipientis*, including it with the RICKETTSIACEAE. The non-pathogenic rickettsiae will not be considered in detail in this paper. For a detailed discussion of them, reference may be made to papers by Wolbach (15), Hertig and Wolbach (20), and Cowdry (16).

The etiological agent of Q-fever, named *Rickettsia burneti* and *R. diaporica*

by Australian and American workers, respectively, although a facultative rather than an obligate intracellular parasite, appears otherwise very closely related to the more characteristic rickettsiae listed above. As a matter of convenience, and with the realization that no entirely satisfactory definition of the rickettsiae can be given at present, this organism will be included with the rickettsiae for the purposes of this discussion. For the same reason and in spite of the fact it is apparently an extracellular organism, the probable etiologic agent of trench fever, *Rickettsia wolhynica* (*pediculi, quintana*), will also be discussed.

*Rickettsia canis*, *R. bovis*, and *R. ovina*, will also be reviewed briefly, for the sake of completeness, since it seems possible that these organisms may eventually be shown to possess the necessary characteristics for inclusion with the rickettsiae.

*Rickettsia-like pathogens* A number of pathogenic agents have been described which resemble the rickettsiae in many ways, but which for one reason or another cannot at present be included with them. The biological properties of these organisms will be discussed briefly at this point, since a study of their relation to each other, to the typical pathogenic rickettsiae, to bacteria, and to viruses brings out facts which harmonize well with modern theories concerning the interrelationship of various groups of pathogens.

*Bartonella bacilliformis* is a facultative, rather than an obligate intracellular parasite, non-filterable, and morphologically and tinctorially greatly resembling the rickettsiae (21). It inhabits the tissues of and is probably transmitted by the bite of sand flies (22, 23), but the mechanism of transmission and the morphological picture of infection in the sand fly have not yet been cleared up (24). This organism has been cultivated on special cell-free media (25), but its close relative, *B. muris*, has resisted such cultivation. The similarity of these organisms to the rickettsiae has been pointed out by various workers (26). Their ability to produce severe anemia, by parasitizing the erythrocytes of their mammalian hosts, together with the lack of information concerning their behavior in the sand fly would appear to justify their exclusion for the present from the rickettsiae. It is of interest to note that *B. bacilliformis*, in plasma tissue cultures, forms extracellular colonies in the plasma clot as well as intracellular colonies (21). This indicates that its growth requirements are simpler than those of the typical rickettsiae (see below).

*Pasteurella tularensis* is a facultative intracellular parasite, non-filterable, and transmitted biologically<sup>1</sup> by ticks. This organism is so perfectly adapted to life in tick tissues that, like the spotted fever rickettsia, it is transmitted hereditarily from one generation to another by infection of the ova. It multiplies in and distends the epithelial cells lining the gut of the tick, producing a picture similar to that of *R. prowazeki* in the louse (26a). In contrast to *R. prowazeki*, however, it also grows freely in the coelomic fluid. The author has found (21) that in tissue cultures it grows both in the cytoplasm of cells and free in the surrounding plasma clot behaving much like *Bartonella bacilliformis*. In guinea

<sup>1</sup> That is, by actually multiplying in tissues, as opposed to purely mechanical transmission.

pigs it behaves rather conspicuously as a facultative intracellular parasite, massively infecting both the Kupffer cells and the liver cord cells (27). The organism has been cultured on special cell-free media (notably those containing cystine) and is of course classed with the bacteria, on the basis of its biochemical reactions. Its similarity to the rickettsiae, although obvious from the above discussion, has not been stressed, and it would serve no useful purpose at present to include it with these organisms.

It has been suggested that the elementary bodies of psittacosis (28) and those of trachoma (29) may be rickettsial in nature. Similar bodies are associated with lymphogranuloma venereum (30), and in all three conditions these structures are confined to the cytoplasm of cells. In the case of trachoma, insect transmission has been suggested (31). Unless insect transmission of these infections is conclusively demonstrated, however, these structures should not be considered as rickettsiae. Their complex morphology, which, in the case of psittacosis has been interpreted as evidence of a life cycle (32), suggests that they may differ considerably from the rickettsiae. Since psittacosis is at present classed as a virus disease, it is customary to refer to the intracellular structures associated with infection as elementary bodies, rather than organisms. The constant association of these discrete bodies with infectivity strongly suggests that they are the etiological agents of the infection, and it is probable that they may prove to be organisms in the same sense that rickettsiae are. If biological transmission by insects should be demonstrated, their inclusion with the rickettsiae would have to be seriously considered.

An intracellular organism associated with conjunctivitis in sheep and goats has been named *Rickettsia conjunctivae* by Coles (33), and a similar organism *R. conjunctivae bovis* was described by the same author in cattle (34). Another similar organism has been described, but not named, by Johnson (35) as the probable cause of a type of conjunctivitis in sheep. It seems unwise to class such organisms as these with the rickettsiae, since the most important single criterion for such assignment—development in insect tissues—has not been established, and it is clear that infection is commonly transmitted by direct contact.

Mochkovski (36) has made the interesting suggestion that the Foa-Kurloff bodies may be rickettsiae. Since he has furnished no proof that these structures are microorganisms, or that they inhabit insect tissues, this suggestion cannot at present be taken seriously.

The rickettsia-like organism described by Sellards and Siler (37) in association with dengue is probably non-pathogenic in nature.

*Interrelationships* For many years the rickettsiae have been considered to represent a new group of microorganisms distinct from bacteria, a point of view which has proven useful and stimulating. From a consideration of the above discussion, the difficulties of drawing sharp lines of demarcation between bacteria, rickettsiae, and viruses become evident. Among rickettsia-like pathogens we have mentioned one (*Pasteurella tularensis*) which is accepted as a bacterium and variously grouped with *Pasteurella pestis* and *Brucella abortus*, and one

(psittacosis "virus") which is commonly classed as a filterable virus. The typical rickettsiae resemble bacteria in their visibility, morphology, and apparent non-filterability, but are close to the filterable viruses in their specificity for certain cell types and in their growth requirements. The modern tendency to regard viruses as organisms which have lost their enzyme systems by a process of evolution tempts one to cast the rickettsiae in the rôle of a missing link.

The inclusion of obligate intracellular parasites with the bacteria is not without precedent, however (for example, the lepra bacillus), and a more satisfactory concept would appear to be that of an unbroken series, with free-living bacteria at one end, the smallest viruses at the other end, and a large number of organisms and elementary bodies intermediate at many points between these two groups. On the whole, therefore, it seems most logical to regard the rickettsiae as bacteria which have become adapted to intracellular life in arthropod tissues. The alternative theory, that bacteria are viruses, originally imprisoned in cells, which have acquired the ability to lead an independent existence, seems less attractive.

In conclusion, it should be again emphasized that the above taxonomic discussion is based on our present incomplete knowledge of biological and morphological properties, and that it is subject to revision with the introduction of new data. Many points are left unsettled. For example, one might ask why rickettsiae could not equally well be regarded as protozoa. The distinction between bacteria and protozoa is made largely on morphological grounds, and the rickettsiae are morphologically closer to bacteria than to protozoa. They lack the internal structure commonly associated with protozoa, and are smaller than organisms classed as protozoa, with the possible exception of certain piroplasmas.

The difficulties encountered in any attempt to classify the viruses are even greater than in the case of the rickettsiae, and much work must be done before the former can be classified, even tentatively, in a reasonably satisfactory way.

The elementary bodies of psittacosis are often referred to as L C L (Levinthal-Cole-Lillie) bodies, but Yanamura and Meyer (38) have recently pointed out that there is no justification for substituting this name for that originally given by Levinthal, namely "*Microbacterium multiforme psittacosis*". Similarly, Goodpasture (39) has proposed the name "*Borreliota variolae hominis*" for the elementary bodies of smallpox. The application of these specific names to the bodies in question indicates a belief that they, like the rickettsiae, are living morphological entities. In the course of time, the true relationship between such bodies as these and intracellular microorganisms such as the rickettsiae will probably become clear. As a matter of convenience, it seems justifiable to give generic, specific, and variety names to such pathogenic agents, even though their family and order relationships are not yet clear.

In discussing the properties of the pathogenic rickettsiae, certain principles will be established first by a detailed consideration of *Rickettsia prowazekii*, the cause of typhus, and the known differences between the other organisms and the type species will be pointed out in the discussion of each.

## RICKETTSIA PROWAZEKI (TYPHUS FEVER)

*Etiological studies.* As mentioned above, the etiologic agent of typhus fever was first described by Ricketts and Wilder in 1910 (6) in lice fed upon typhus patients. Further evidence that the organism described by Ricketts is etiologically related to typhus was furnished by da Rocha-Lima (4) who named the organism *Rickettsia prowazeki*. The work of Wolbach, Todd and Palfrey (7), carried out under ideal experimental conditions, entirely confirmed the etiologic concept previously formulated by Ricketts and da Rocha-Lima. Wolbach and his co-workers showed that lice, originally free from rickettsiae, acquired numerous rickettsiae which distended the intestinal lining cells, when fed on typhus patients. These workers also demonstrated intracellular rickettsiae in the lesions of man and experimental animals. When the intestines of lice, containing intracellular rickettsiae, were emulsified and injected into guinea pigs, typhus fever resulted, whereas injection of the intestines of rickettsia-free stock lice produced no illness. Considering the intestinal tract of the louse as the equivalent of a bacteriological culture medium, it could be said at this time that, in a sense, Koch's laws had been fulfilled.

In spite of the conclusive nature of the above described work, skepticism regarding the etiology of typhus persisted for many years. This was due partly to the fact that the experimental work was of such a nature that it could not be readily repeated, and partly to the difficulty of demonstrating *R. prowazeki* in mammalian tissues. These organisms were never demonstrable in large numbers, and, because of their small size and slight affinity for aniline dyes, could be demonstrated only in perfectly fixed and stained preparations, studied with great patience.

Neill (40) found that in guinea pigs reacting to Mexican typhus after intraperitoneal inoculation, an acute fibrinous exudate formed in the scrotal sac, while the general peritoneal cavity showed no such reaction. Later, Mooser (41) found that many cells distended with rickettsiae were constantly present in this exudate. The free growth of the organism apparently depends upon the lower temperature obtaining in the scrotal sac. With the advent of this new rickettsia-rich material to work with, and of better methods for staining rickettsiae in sections, progress was more rapid.

Further confirmatory evidence of the etiologic relationship of *R. prowazeki* to typhus has been furnished by the cultivation of the organism within living or surviving cells in artificial media of various types (see below). In plasma tissue cultures, not only was the presence of rickettsiae accurately correlated with infectivity, but the incubation period of the induced infection was found to be roughly proportional to the number of organisms present in the cultures, ranging from 48 hours up to 25 days (42). Zinsser and Castaneda (43) showed that rickettsiae, freed from cells and washed repeatedly at the centrifuge, were still capable of producing typhus. On the whole, it seems safe to state that the etiologic relationship of *R. prowazeki* to typhus is as well established as, for example, that of the tubercle bacillus to tuberculosis.

The purely theoretical concept that the etiologic agent of typhus may exist in three forms, the bacterial, the rickettsial, and the invisible, will be found frequently in early literature, and has even been sponsored more recently by such authority as that of Nicolle (44). The bacterium most commonly listed is *Proteus vulgaris*. The process of "evolution" devised as an explanation of the variation in form does more credit to the imagination of its proponents than to their critical judgment. The etiologic relationship of certain strains of *P. vulgaris* to typhus was naturally suggested by the high titer in which it agglutinated by typhus serum (see below). Claims that strains of *P. vulgaris* or other organisms cultivated on bacteriologic media have produced typhus fever in guinea pigs do not stand critical analysis.

The contention that the etiologic agent of typhus may exist in an invisible form is somewhat more difficult to disprove. A similar concept has been entertained for spotted fever (45). In no instance, however, has any direct proof for such a theory been advanced. The fact that material containing no demonstrable rickettsiae may be infectious is evidence of an inconclusive nature, since a few dozen rickettsiae in a cubic centimeter of fluid would be practically impossible to find and recognize. In the author's work with Hass (42), typhus tissue cultures were bisected, one half being embedded and sectioned for microscopic study and the other half injected into guinea pigs. In the great majority of cases, when infection occurred in guinea pigs as a result of the injection of one half of the culture, rickettsiae were found in paraffin sections of the other half. In a very few instances, cultures gave a positive inoculation test while no organisms were seen. In such instances, however, the incubation period of the induced infection was invariably greatly prolonged, suggesting that the number of organisms present may have been small enough to escape detection. These experiments indicated the improbability of invisible forms of typhus and spotted fever rickettsiae occurring under these conditions. The evidence for an invisible form of the etiologic agents of typhus and spotted fever under any other conditions is on the whole meagre and inconclusive.

*Size, filterability, morphology, and staining.* The smallest dimension of *R. prowazeki* is commonly given as 300 m $\mu$ , but forms which appear smaller than this and are almost on the borderline of visibility are occasionally seen, both within cells and outside of cells (freed by rupture of the cells). Even if we accept the figure of 300 m $\mu$ , these organisms would have approximately the same size as the elementary bodies of psittacosis (estimated at 250 to 275 m $\mu$ ). The latter are filterable, while rickettsiae are apparently non-filterable. Typhus rickettsiae rapidly lose virulence when freed from their host cells, and it is the author's opinion that the question of filterability needs further study under carefully controlled conditions. Isolated rickettsiae, freed from all remnants of cell cytoplasm and suspended in a medium in which they remained virulent for the duration of the experiment, would have to be employed for this purpose. So far as the author is aware, these conditions have not been satisfied. The rickettsiae of Q-fever, which appear somewhat larger than typhus rickettsiae, readily pass through Berkefeld N filters (46). The question of the filterability of

*R. prowazeki* is a matter of arbitrary definition and subject to some uncertainty, since the organism is obviously of such a size that it might, under certain conditions, prove filterable

As seen in paraffin sections, *R. prowazeki* is always intracellular. In smears, extracellular organisms are often seen, as a result of the rupture of cells in the process of making the preparation. The organism exhibits considerable pleomorphism, but this factor has perhaps been over-emphasized. In its most characteristic form, it appears as a minute diplobacillus, each member of the diploid form averaging about  $0.6 \times 0.3 \mu$ . Diploid forms may at times appear as a single organism, because the space between the two organisms may not be seen. Short chains are not uncommon. Occasionally, in recently infected cells in tissue culture, long chains ranging up to  $40 \mu$  in length may be seen. Wolbach and his co-workers (7) described similar forms in lice, in the early stages of infection "within non-swollen cells of the midgut at a time when very few cells are infected with the coccoid rickettsia." These long chains apparently develop under optimum conditions of nutrition only in the early stages of cell infection, and it seems unlikely that they represent a stage in a life cycle.

In very heavily infected cells, which may become greatly distended, the individual organisms appear as minute coccoid bodies, usually in diploid formation. These coccoid forms approach the limit of the resolving power of the ordinary microscope. That these minute forms are not an optical illusion, caused by the packing in the cells, is shown by the fact that they can occasionally be seen extracellularly when a distended cell has ruptured. Rather than to consider these forms as evidence of a life cycle, the author prefers to regard them, like the chain forms, as morphological variants dependent on nutritional factors. The possibility that variations in cellular immunity may be a factor in causing this pleomorphism, as has been suggested for the bodies of psittacosis (38), cannot be ruled out, of course.

*Rickettsia prowazeki* stains poorly with most aniline dyes. The organism is gram-negative in the sense that it cannot be recognized with certainty when stained by the gram method, and obviously does not retain the dye. In film preparations, the organism can be stained very satisfactorily by the Giemsa method, or by Macchiavello's modification of Cantaneda's stain (47). In the former method, the rickettsiae are stained blue or purple, while the cell cytoplasm is usually a pale blue. By the latter method, the rickettsiae are stained red and stand out sharply against a blue cytoplasmic background. In paraffin sections, the Giemsa method, after Regaud's fixation (48), stains the organisms very clearly, but the use of this method requires some experience.

The faint staining of the organism, particularly as applied to sections, has been exaggerated. It is quite true that in Giemsa-stained films rickettsiae are commonly pale as compared to staphylococci, for example, but in paraffin sections they may be stained by various methods as deeply as may be desired, and when overstained their apparent size is greatly exaggerated.

*Cultivation, and growth requirements.* Typhus rickettsiae have not been cultivated in the absence of cells. They grow freely in the medium containing

living or surviving cells which have been used for virus cultivation, namely, plasma tissue cultures, the Maitland medium, and the Zinsser-Wei-FitzPatrick medium

Experiments by Wolbach and others (49) showed that *R. prowazeki* could multiply in a restricted way in plasma tissue cultures of mammalian cells at 37.5 C. Massive infection was not obtained in this early work. Nigg and Landsteiner (50) reported the unrestricted intracellular multiplication of the organism in a modified Maitland medium, consisting of minced guinea pig tunica vaginalis suspended in a mixture of serum and Tyrode's solution. Pinkerton and Hass (42, 51) reported similar massive multiplication of the organism in plasma tissue cultures grown at 32 C. In the Nigg-Landsteiner medium, multiplication was equally good at 32 or 37.5 C, but in the plasma tissue cultures the lower temperature was found to be essential.

The Zinsser-Wei-FitzPatrick medium (52) is essentially a modified Maitland medium, the serum-Tyrode mixture being solidified by adding agar, and the tissue (chopped mouse or chicken embryo) spread on the surface. The tissue fragments are more thoroughly bathed by the fluids in this medium, and the method represents an important improvement over previous similar methods.

In smear preparations from cultures of the above types, one finds large numbers of extracellular organisms, suggesting extracellular multiplication. When identical preparations are studied in paraffin sections, however, the evidence is clear that multiplication is intracellular, and that extracellular organisms have recently been set free by the rupture of distended cells during the preparation of the film. The contents of a single cell, packed and distended with rickettsiae, may cover a large area if the organisms were spread out only one layer deep, much as a minute drop of blood will make a film over the entire surface of a coverslip.

Although their growth requirements are in general similar to those of the typical viruses, an interesting difference has been brought to light. The author found that in plasma tissue cultures typhus rickettsiae died rapidly at 37 C, and grew best at 32 C (53). This behavior is probably to be correlated with the fact that certain strains, after intraperitoneal injection in the guinea pig, grow more readily in the scrotal sac, where the temperature is lower, than in the general peritoneal cavity, and produce in the scrotal sac a fibrinous exudate containing large numbers of heavily infected cells (the Neill-Mooser reaction described above). In the Maitland medium, rickettsiae multiply equally well at 32 C and 37.5 C. This apparent discrepancy is probably to be explained on the basis of the different conditions obtaining in the Maitland medium and in plasma tissue cultures. In the former, the cells are surviving rather than actually living, and mitotic division of the cells is rarely seen, while in plasma tissue cultures the cells multiply rapidly and the metabolic rate of the cells is probably higher. From these facts the conclusion was drawn (54) that typhus rickettsiae grow best in cells which are metabolizing slowly. Zinsser (55) has recently confirmed this and pointed out that certain viruses, in contrast to rickettsiae, grow best in healthy, actively metabolizing cells.

Still further confirmation has come from the observation by the author with

Bessey (56) that riboflavin deficiency, a condition which lowers intracellular metabolism by interference with the formation of the yellow respiratory enzyme, causes marked loss of resistance to typhus infection in the rat, allowing the rickettsiae to multiply profusely in the reticulo-endothelial cells in various organs, and resulting in a fatal disease. A similar specific lowering of resistance to psittacosis as a result of thiamin deficiency has been reported by the author with Swank (57). A smaller, and presumably more perfectly adapted virus, however, (the Lansing strain of poliomyelitis), has been found (58) to cause equally severe illness in normal and riboflavin-deficient mice, with an apparently higher mortality rate and somewhat earlier death in the normal than in the deficient animals. Similarly, the Rous chicken sarcoma grows much more slowly in riboflavin-deficient than in normal chickens, the deficient birds outliving the controls by several weeks (38). Thus it appears that certain viruses, in contrast to typhus rickettsiae, are inhibited by riboflavin deficiency in their host.

Study and speculation have led various workers to regard intracellular parasitism as primarily an adaptation to intracellular existence, associated with an acquired ability to utilize intracellular enzymes of the host cell, and with a corresponding loss (by disuse?) of enzymes on the part of the parasite. In a general way, but not necessarily with any degree of accuracy, this loss of enzymatic complexity may be correlated with a diminution in size of the parasites. From this point of view the rickettsiae are of particular interest, since they are intermediate in size between most free-living bacteria and the smaller viruses, and, from the above observations regarding their growth requirements, might also be regarded as intermediate in their degree of adaptation to intracellular conditions, since they grow best in cells which are metabolizing slowly.

Further study is needed, however, before we can safely generalize regarding the metabolic activity of the viruses. It is not inconceivable, for example, that some of the smaller viruses may be shown to multiply most freely in slowly metabolizing cells, or even to approach the free-living bacteria in their metabolic requirements. On the other hand, it is of interest to note that a number of protozoa, for example "*Toxoplasma*," although large in size and complex in structure, behave in mammalian tissues as obligate intracellular parasites (59).

The absence of a single important metabolic activator might be as effective in preventing the cultivation of an organism in cell-free media as the absence of a number of enzymes. The problem in each intracellular parasite, is to find out exactly what activators are lacking. Presumably, if the missing enzymes or other activators could be supplied, together with suitable food substances and physical conditions, any obligate intracellular parasite could be cultured without living cells. It should also be pointed out, however, that parasites within cells may be protected from certain toxic substances which prevent their multiplication outside of cells.

The study of rickettsiae in plasma tissue culture affords a logical approach to the question of their growth requirements, both physical and metabolic.

As mentioned above, typhus rickettsiae disappear rapidly from their host cells in tissue cultures maintained at 37.5 C, and the cultures are no longer

infectious for guinea pigs after about the tenth day. At 42 C, the rickettsiae disappear even more rapidly from the cells. At 27 C, the cells die in about two weeks, and the rickettsiae disappear. At 32 C, however, the rickettsiae find optimum conditions for intracellular growth, and practically every cell becomes distended with them (53). These heavily parasitized cells are apparently uninjured and are still capable of mitotic division. Such cultures remain heavily infected for several months when maintained at 32 C, but when placed at higher temperatures rapidly become free of rickettsiae and lose their infectivity. This striking dependence on temperature is probably indirect rather than direct. The organisms grow luxuriantly at 32 C not because they prefer that temperature, but only because that temperature is most effective in bringing about suitable growth conditions within their host cells. These growth conditions, as in the case of riboflavin deficiency, probably result from a lowering of the intracellular metabolism.

Alterations in the partial pressure of oxygen in such cultures have been shown to be without effect on the intracellular growth of typhus rickettsiae, unless the concentration of oxygen becomes so low that the cells die. In this case, the rickettsiae also soon die, since they are unable to live within dead cells. Similarly, alterations in the pH of the tissue culture medium are without effect except insofar as they may cause the death of the cells.

It is probable that such alterations in pH of the medium do not appreciably change the intracellular pH. Attempts to alter the pH of the cell cytoplasm by organic acids and bases ( $\text{CO}_2$  and  $\text{NH}_3$ ) were, however, likewise without apparent effect on the intracellular multiplication of rickettsiae (60).

Typhus rickettsiae in tissue culture not only fail to multiply extracellularly in the plasma clot, but apparently survive only for brief periods when set free from disintegrating cells. This statement is based on the fact that, even in cultures where cells are allowed to disintegrate, organisms are seen in the plasma clot only in extremely small numbers, and never at a great distance from their host cells (51).

Another method of approach to the problem of the metabolism of rickettsiae is represented by attempts to cultivate them in symbiosis with free-living bacteria, either in direct contact with these organisms or near enough, on solid media, to permit the diffusion of nutrient substances, on the theory that the enzyme systems of the free-living organisms might be utilized in some way by the rickettsiae. Although the possibilities have by no means been exhausted, a considerable number of experiments of this type have been entirely negative.

On the whole, then, it can be said that little definite information is available regarding the actual metabolism of rickettsiae and other intracellular parasites, although the rickettsiae seem to be particularly suitable for the study of this problem.

*Cytological studies* Rickettsiae have the advantage over the smaller viruses as objects for cytological study of being clearly visible as individuals in their host cells. They can be clearly seen in unstained living cells with either dark or light field illumination, and their behavior under various conditions can be

readily observed. In the early stages of their intracellular multiplication, typhus rickettsiae lie motionless in an apparently gelatinous cytoplasm, surrounded by only a narrow clear halo. Later, the organisms are grouped in what appear to be fluid vacuoles and move freely within these vacuoles by Brownian movement (60). True motility has not been observed, either within or outside of cells. As infected cells disintegrate, small groups of rickettsiae, moving freely within these small spherical fluid vacuoles, are set free and float out into the surrounding medium. This mechanism is probably important in the spread of infection from cell to cell.

Typhus rickettsiae multiply exclusively in the cytoplasm of cells in tissue cultures, and are never seen in nuclei. This behavior is in striking contrast to that of spotted fever rickettsiae (61). Since typhus rickettsiae would, like spotted fever rickettsiae, probably be occasionally included within nuclei, as the nuclear membrane is reformed after mitotic division, one can only conclude that conditions within the cell nuclei are for some reason incompatible with the life and growth of typhus rickettsiae. The reason for this difference in behavior between the two organisms has not been ascertained.

The behavior of typhus rickettsiae during mitotic division of their host cells is interesting. The organisms are markedly reduced in number during this process, and tend to collect at the poles of each cell (51). They also, in many instances, assume a more spherical shape. The period of mitotic division, perhaps because of the increased metabolic rate within the cells, is apparently unfavorable for the rickettsiae, and many of them die at this time. Those which survive, however, rapidly multiply until they distend the daughter cells.

When fat droplets appear within cells in tissue culture, the rickettsiae are never seen within these droplets, but they crowd the cytoplasm around the droplets.

The possible relationship of rickettsiae to mitochondria has frequently been a subject for speculation. Their morphology and staining reactions are not inconsistent with such a viewpoint, but Cowdry and Nicholson (62), after staining mitochondria and rickettsiae alternately in mammalian cells, concluded that the two types of structures were separate and distinct. The fact that rickettsiae may distend cells, and in the case of spotted fever even multiply within nuclei where mitochondria have never been described, is also evidence of their different nature. It seems quite clearly established that rickettsiae, as seen in parasitized mammalian cells, are unrelated to the mitochondria of these cells. In view of the similarity of certain rickettsiae to the intracellular symbionts of insect tissues and the lack of a sharp line of demarcation between the latter and mitochondria, the question of a relationship between rickettsiae and the mitochondria of insect tissues should perhaps not be considered as finally settled. Although it would be entirely possible to do so, no one has actually watched rickettsiae enter cells and observed their actual multiplication after entering. By this simple observation, the question of their intracellular origin could be definitely settled.

*Viability under various environmental conditions* *R. prowazeki* is readily killed by heat, drying, or chemical agents. A few minutes at 50 C suffice to destroy the virulence of blood and tissue emulsions, while such materials when dried under ordinary conditions at room or incubator temperatures become non-virulent in a few hours. Blanc and Baltazard report, however, that virulence is retained in the thoroughly dried feces of infected fleas for periods up to 651 days.

Any discussion of the viability of the organisms must take into consideration the medium and the protective action of the cytoplasm of the host cells. As has been brought out above, rickettsiae appear, under all conditions in which they have been studied, to survive for only a few hours when freed from this protection. The supernatant fluid from heavily infected Maitland cultures of typhus rickettsiae is non-virulent when injected into guinea pigs. In defibrinated typhus blood, virulence is lost in about 72 hours at incubator temperatures and in less than four days at room temperature. Scrotal sac exudate, containing many intracellular typhus rickettsiae, was found by the author (63) to lose virulence between the third and eighth days when suspended in glucose broth at room temperature.

Rickettsiae are commonly said to be, like many of the viruses, resistant to the action of glycerine. Strictly speaking, however, it is probably not a question of resistance to this substance, since the glycerine probably does not come in contact with the intracellular organisms. Glycerine probably helps to preserve the viability of the organisms by virtue of its dehydrating action on their host cells. Infective typhus guinea pig tissues, suspended in glycerine, can retain their virulence for several months at 0 C (63), while the same tissues suspended in physiological salt solution or merely sealed in test tubes lose their virulence in a few days under these conditions.

At -20 C, however, a brain or spleen from a typhus guinea pig, merely sealed in a test tube, retains its virulence with a gradual loss of titre for periods ranging up to eight months (63). Scrotal sac exudate, minced and suspended in Tyrode's solution, becomes non-virulent in two weeks at -20 C, but if suspended in a mixture of equal parts of blood serum and Tyrode's it retains virulence for periods up to six months (63). Topping (64) finds that typhus rickettsiae remain virulent for several months (end-point not determined) in tissue dried in the frozen state in the "lyophile" form or by the "Cryochem" process.

Nigg (65) has reported that typhus rickettsiae in sealed Maitland cultures at 37 C are virulent for periods ranging up to several months. Although the host cells undoubtedly die under these conditions, they do not disintegrate and apparently continue to maintain the rickettsiae in a viable state. As has been said, defibrinated blood from a typhus guinea pig loses its virulence in a few days at incubator temperatures.

On the whole, the behavior of typhus rickettsiae under various environmental conditions is similar to that of many viruses. In both cases, the environmental conditions which preserve virulence are probably those which tend to maintain the integrity of the cytoplasm of the host cells. The conditions under which extracellular rickettsiae retain their virulence have not been sufficiently studied.

*Pathology, and pathogenicity for mammals* The histopathology of typhus infection need not be discussed in detail here. Suffice it to say that the characteristic pathologic changes are brought about by the multiplication of *R. prowazeki* within the endothelial cells of the small blood vessels throughout the body, but particularly in the skin and brain. The pathology is entirely similar in man and in experimental animals.

Among the lower animals, monkeys, guinea pigs, dogs, cats, rabbits, rats, mice, gophers, woodchucks, jackasses, ground squirrels, and flying squirrels are susceptible in varying degrees to typhus infection. In many of these animals, no fever or other outward manifestation of infection is seen, but the infection persists for long periods (up to 370 days in white rats) in an inapparent form.

The most satisfactory animal for the laboratory study of typhus is the guinea pig, in which both murine and human typhus causes a febrile but non-fatal illness. The differences in the scrotal reaction which are of importance in distinguishing between the two strains of typhus, will be discussed below. The scrotal reaction occurs only after intraperitoneal injection, but a febrile illness and the characteristic focal brain lesions are produced equally well by subcutaneous inoculation.

*Strain variation* An outstanding characteristic of the pathogenic rickettsiae is their tendency to occur in strains of widely different virulence, both for man and for experimental animals. The possible relation of the pathogenic rickettsiae to certain of the non-pathogenic rickettsiae in arthropods requires further study, but the evidence at present is against any close relationship (13).

In addition to strain variations in virulence, however, careful study has brought to light interesting biological variations between strains of *R. prowazeki*. These differences are more or less permanent modifications, probably caused by prolonged residence in different species of arthropods and in different species of mammals.

Two distinct types of *R. prowazeki* are recognized. The differences between these two types were believed by the author (18) to be of sub-specific magnitude, and consequently the two types may be given the variety names *R. prowazeki* var. *prowazeki* and *R. prowazeki* var. *mooseri*. The former is the etiological agent of louse-borne human typhus, of the type which has occurred in great epidemics on the European continent and has been called European or epidemic typhus. *R. prowazeki* var. *mooseri* is the name applied to the etiological agent of a somewhat milder, usually endemic, form of the disease occurring in various parts of the world, and apparently enzootic in rats. This form of the disease is carried from rat to rat by the rat louse (and by the rat flea) and from rat to man by the rat flea. It is best called murine typhus, a name suggesting the importance of the rat in its epidemiology. According to Zinsser, both forms of the disease may occur in epidemic or endemic form, and the murine type in Mexico can, like the human type, be carried from man to man by lice.

The differences between these two varieties of *R. prowazeki* may be summarized as follows:

1. *R. prowazeki* var. *mooseri* after intraperitoneal injection in rats causes a febrile illness, while *R. prowazeki* var. *prowazeki* produces only an inapparent

infection Male rats infected with *R prowazeki* var *mooseri* suffer a mild inflammation of the tunica vaginalis with many serosal cells laden with rickettsiae while *R prowazeki* var *prowazeki* causes no such reaction

2 *R prowazeki* var *mooseri*, when introduced *per anum* into *Pediculus humanus*, kills this arthropod in a few days, while *R prowazeki* var *prowazeki* requires about three weeks This phenomenon is probably independent of the number of viable organisms injected

3 After intraperitoneal injection in male guinea pigs, *R prowazeki* var *mooseri* almost constantly induces an acute inflammation of the scrotal sac with numerous visible organisms, while *R prowazeki* var *prowazeki* either fails to cause such a reaction at all or produces it irregularly and in a milder, more transient form (66)

4 Zinsser's studies with Castaneda (67) brought out certain minor immunological differences between the two varieties, which will be discussed below

5 Perhaps most important of all distinguishing features from a practical point of view is the fact that *R prowazeki* var *prowazeki* grows much less luxuriantly, not only in living experimental animals with resistance reduced by various methods, but also in the various media containing living or surviving cells This fact makes the production of vaccine, in practical quantities, a more difficult problem than in the case of *R prowazeki* var *mooseri* Further discussion of this phenomenon with reference to immunological problems will be found below

These differences between the two varieties of *R prowazeki* seem at first glance to be relatively trivial, but careful study has shown that they are constant and important Nobody has succeeded in changing one variety into the other, even after years of passage in different species of animals Human typhus rickettsiae, which have been producing only an inconspicuous scrotal reaction in guinea pigs, can be made to produce a severe scrotal reaction, comparable to that seen in murine typhus, by passing the infection through rats and then back to guinea pigs (68) In subsequent transfers, however, the scrotal reaction reverts to its mild, inconspicuous type It is, however, believed, without anything like conclusive proof, that with crowding together of human beings and an abundance of lice, the severe, epidemic infection with *R prowazeki* var *prowazeki* may evolve in months or years from a single case of human infection with *R prowazeki* var *mooseri* acquired directly from a rat by the bite of a rat flea There is, on the other hand, some reason for believing that *R prowazeki* var *prowazeki* may be endemic in man in certain parts of the world, and if so, important epidemics may start directly, without the necessity of a process of transformation of one variety into the other

Zinsser (69) has introduced strong evidence that cases of typhus in the North Atlantic states are late recrudescences of childhood infection with *R prowazeki* var *prowazeki*, acquired in Europe If this be the case, the survival of *R prowazeki* var *prowazeki* during interepidemic periods, quite independent of the murine form of the disease, could readily be explained

*Immunology* Infection with the rickettsial diseases usually confers long-

lasting immunity The study of the problem of active immunization in typhus and spotted fever has centered about the development of methods for the accumulation of rickettsiae in sufficiently large concentration to be of practical value Ordinarily, the concentration of rickettsiae in the tissues of infected laboratory animals is too low to be of value Tissue vaccine, therefore, of the type made by Laidlaw and Duncan for immunization against distemper, is of no value

Weigl (70) took the first step in 1930 toward a solution of this problem by using the heavily parasitized intestinal tissues of lice, infected by introducing the virus *per anum*, and subsequently maintained on typhus-immune human beings Although of proven value, this method of vaccine production cannot be utilized on a large scale because it is too time-consuming

Benzol poisoning, perhaps because of its destructive effect on the bone marrow, was found by Zinsser and Castaneda to increase the number of rickettsiae in the peritoneal lining cells of rats infected intraperitoneally with murine typhus Better and more constant results were later obtained by subjecting the rats to suitable doses of x-rays (71) Probably because of the low natural susceptibility of the rat, this method is not effective for human typhus The "agar tissue culture" method of Zinsser and co-workers has already been described

*R. prowazeki* was grown on the chorio-allantoic membrane of the developing chick embryo by Zia (72) The concentration of rickettsiae obtained by this method was not great enough, however, to justify its use for vaccine production Cox showed that much higher concentrations of rickettsiae could be obtained by injecting the organism into the yolk sac of the developing egg embryo (73) Smears of such preparations showed such large numbers of apparently extracellular organisms that for a time it was believed that extracellular multiplication was taking place It is now known, however, that multiplication is entirely within the lining cells of the yolk sac, and that here, as under all other known conditions, *R. prowazeki* behaves as an obligate intracellular parasite

The Zinsser-Wei-FitzPatrick medium (52) and the method of Cox both have the advantage over other methods of being applicable to human typhus (*R. prowazeki* var *prowazeki*) and spotted fever (*D. rickettsi*) as well as to murine typhus

The author, with Bessey (56) obtained large concentrations of *R. prowazeki* var *mooseri* by the intraperitoneal injection of murine typhus rickettsiae in riboflavin-deficient rats The possibilities of this method for vaccine production have not been exploited No success was achieved by this method with either human typhus or spotted fever rickettsiae, probably because of the high natural resistance of the rat to these agents It is possible that the adaptation of this method to guinea pigs, which are highly susceptible to human typhus and spotted fever, might give results of practical value

Castaneda (74) has discovered still another method for producing high concentrations of *R. prowazeki* var *mooseri*, namely, the intratracheal injection of the virus in rats and mice He believes that the murine strain is antigenically

broadier than the human, and that successful vaccination against the latter can be achieved by the former if large doses are given. If this proves to be so, the problem will be simplified, since high concentrations of *R. prowazekii* var *mooseri* are relatively easily obtained by all known methods.

Further work is necessary to decide on the relative merits of the various ingenious methods of vaccine production outlined above, but at present the yolk sac method of Cox has probably the greatest demonstrated practical value and is being utilized most extensively. Even in the face of continued failure to cultivate typhus rickettsiae on bacteriologic media, the outlook for large-scale vaccine production is by no means dark.

*Passive immunization* By injecting very large quantities of rickettsiae into a horse over long periods of time, Zinsser and Castaneda (75) have prepared an immune serum of considerable potency for guinea pigs infected with both human and murine typhus. The practical value of such serum for human patients has not been demonstrated.

*Living virus immunization* Laigret and co-workers (76) have developed a method of immunization based on the injection of guinea pig tissues containing the infective agent of murine typhus in an attenuated form. Such methods are effective only insofar as they introduce living rickettsiae and cause actual infection. The dangers of such procedures are obvious, and a few fatalities have occurred from their use in Chile. It cannot be denied, however, that under certain conditions, as in the face of devastating epidemics, the production of the relatively mild murine typhus in the population on a large scale might be a justifiable emergency measure. Experiences during the present war will no doubt give important information regarding the value of various prophylactic measures.

Vaccination with mixtures of living rickettsiae and immune serum (Zinsser and Macchiavello, (77)) should also be mentioned in this connection. The difficulty of this method is in controlling accurately the balance between the serum and the living organisms.

*Agglutination reactions* Although the agglutination of certain strains of *Proteus vulgaris* by sera of patients infected with typhus and spotted fever has been used for many years as a diagnostic procedure, no true biological relationship between that bacterium and the rickettsiae has been demonstrated. Castaneda (78) believes that the phenomenon depends on the possession of a common carbohydrate antigen by the two organisms. The titre of agglutination is unusually high for a non-specific reaction, but, however closely related the two organisms may be antigenically, they have in their present forms nothing in common except the probable possession of a common antigen. Uncritical experiments suggesting that *P. vulgaris* may cause typhus fever are not worthy of serious consideration.

In typhus, agglutination is usually found in relatively high titre to the OX 19 strain of *P. vulgaris*, and in low titre with the OX K strain. This fact is of value in differentiating typhus from *tsutsugamushi* disease, since in the latter the agglutination titre is characteristically high with OX K and low with OX 19.

In spotted fever, agglutination in relatively low titre with both OX K and OX 19 is the rule (18)

Agglutination tests can also be carried out with suspensions of *R. prowazeki* of both murine and human varieties, obtained and concentrated by any of the methods described above. Cross agglutination tests carried out by Zinsser and Castaneda (67) showed much higher agglutination titres with the homologous rickettsiae than with the rickettsiae of the other strain. Immune serum also protected better against the homologous infection.

Complement fixation tests have been shown to be positive in typhus (79)

*Latent infection* On the whole, the tendency of the rickettsiae to cause latent infection is somewhat less impressive than that of many viruses. The brains of rats may harbor the rickettsiae of typhus for as long as 370 days (80). The evidence introduced by Zinsser (69), although strongly suggesting that latent infection may persist in man for many years, should probably not be accepted without further confirmation. Attempts to prove that thromboangitis obliterans is a late manifestation of typhus (81) deserve only casual mention, since no valid evidence to support this view has been introduced.

*Chemotherapy* Kikuth (82) has recently stated, but without specific data, that the sulfonamides exert a definite chemotherapeutic effect in rickettsial diseases. Topping (83) obtained negative and even suggestively detrimental effects in typhus-infected guinea pigs by the administration of prontosil and sulfapyridine. Chemotherapy seems as yet to offer little promise, and its use in typhus may even be dangerous.

*Methods of diagnosis* Sporadic cases of rickettsial disease are of frequent occurrence in various parts of the world. Many of these cases are clinically so mild or atypical that special laboratory tests are necessary to establish a diagnosis. In the majority of such cases, the intraperitoneal injection of 5 ml of the patient's blood into each of two guinea pigs during the first few days of illness will result in a febrile disease with involvement of the scrotum or scrotal sac. This phenomenon, which is the result of rickettsial growth in a region of lower temperature, is almost diagnostic of typhus or spotted fever. By killing a guinea pig and making smears from the scrotal sac, a presumptive diagnosis can usually be made, since in typhus we find cells packed with rickettsiae, while in spotted fever the organisms are fewer and larger, and tend to have the characteristic lanceolate form already described. Conclusive proof of the nature of the infection is usually obtainable by carrying out cross-immunity tests with known strains of typhus and spotted fever rickettsiae.

In certain cases, very mild strains may be encountered, which do not become manifest until transfers are made to a second series of guinea pigs. Certain strains may even be difficult to maintain in guinea pigs and cross-immunity tests may be ambiguous. Tissue culture studies, with the application of the criterion of intranuclear *versus* intracytoplasmic growth (18) may be necessary to establish a diagnosis in such cases.

In order to establish a diagnosis during convalescence cross-protection tests with the patient's serum may be carried out. The various diagnostic methods

have been described in detail by the author (18) Animal inoculation is a simple procedure and should be utilized more frequently than it has been in the past

#### DERMACENTROXENUS RICKETTSI (SPOTTED FEVER)

*Dermacentroxenus rickettsi* is the etiological agent of a number of diseases of the spotted fever group Although similar in many respects to *Rickettsia prowazeki*, important differences exist which fully justify its assignment by Wolbach (17) to a different genus All diseases of this group are transmitted by various species of ticks Originally, the disease was believed to be sharply localized to the general region of the Rocky Mountains, and was called Rocky Mountain spotted fever It has now been demonstrated that essentially similar diseases, often showing minor clinical variation and transmitted by different species of ticks, occur in nearly all parts of the world

*Etiological studies* The development of our knowledge of the etiology of spotted fever parallels in many ways that of our knowledge of the etiology of typhus Ricketts (84), and King (85), independently announced in 1906 the transmission of spotted fever to guinea pigs by ticks Ricketts also demonstrated the hereditary transmission of the infection in ticks He undoubtedly saw the etiological agent of the disease in the tick but did not differentiate it clearly from non-pathogenic organisms of similar appearance He noted, however, the occurrence of large numbers of organisms in the ova of ticks, and showed that these organisms were agglutinated by immune serum from human beings and guinea pigs Ricketts also described the organism, which he likened to the influenza bacillus in appearance, in blood smears from guinea pigs and man, but this observation has never been confirmed

Wolbach (17) published in 1919 the results of his thorough and careful etiological and pathological studies, and named the etiological agent *Dermacentroxenus rickettsi* Wolbach differentiated between this organism and non-pathogenic organisms in ticks, and was the first to demonstrate the intranuclear multiplication of the organism in tick tissues

Confirmatory evidence of the etiological relationship of the organism to the disease was furnished by its cultivation in tissue culture In mammalian cells grown *in vitro*, the presence of the organism is accurately correlated with infectivity (61) The same is true of infected tick tissues, but here, except for the intranuclear localization of the organism, it is difficult to distinguish it from non-pathogenic organisms (13) In tissue cultures initiated from the tissues of infected guinea pigs, this difficulty does not arise

Parker (45) believed that the etiological agent of spotted fever could occur in an invisible form, and that the "virus" could exist in ticks in an inactive form which may become active when the ticks ingest blood He has found high titres of infectivity in ticks free from rickettsiae demonstrable by smears, and has found rickettsiae in ticks which were not infective It seems probable that rickettsiae in non-infective ticks are the non-pathogenic rickettsiae which are often found in ticks, and which may closely resemble *D. rickettsi* morpho-

logically These non-pathogenic rickettsiae are never found within cell nuclei, however, and are believed to be distinct from *D rickettsi*. The failure to find rickettsiae in smears of infective ticks may be explained by the fact that the smear method does not demonstrate intranuclear forms. In paraffin sections, cytoplasmic rickettsiae may be absent while thousands of nuclei contain many rickettsi. In tissue cultures, infectivity was never found in the absence of demonstrable rickettsiae. It is the author's opinion that all the observed phenomena can be explained without assuming the existence of the etiological agent of spotted fever in an invisible form.

Parker's observation (45) that hibernating ticks contain the etiological agent of spotted fever in a non-infective or merely immunizing form, which changes to a highly virulent form when these ticks are fed, has never been satisfactorily explained. It has not been established, however, that infection depends on factors other than the concentration of viable rickettsiae.

*Size, filterability, morphology, and staining* The smallest dimensions of *D rickettsi* are about the same as those of *R prowazeki*, and material passed through Berkefeld filters has never been shown to be infectious. The determination of size by filtration experiments, in addition to the usual uncertainties of this procedure, is made difficult by the fact that the organisms, when freed from cells, rapidly become non-virulent unless suitable physical conditions are provided. Wolbach (17) found that thoroughly crushed tissues of infected ticks, suspended in salt solution, were non-virulent.

*D rickettsi*, under certain conditions, shows a striking morphological resemblance to *R prowazeki*. As seen in the scrotal sac of infected guinea pigs, differentiation on morphological grounds is extremely difficult. The most definite difference is the occurrence in spotted fever of paired forms, the individual members of which have tapered ends, so that they somewhat resemble small pneumococci. This form was first described by Wolbach (17) and was believed by him to be a resting stage, while the bacillary forms, of which he described a large and a small form, were believed to be multiplicative stages.

As seen in tissue cultures (61, 54), where an excellent opportunity is afforded for morphological study owing to the heavy infection and perfect fixation of the small tissue fragments, the morphological range of *D rickettsi* is certainly greater than that of *R prowazeki*. The large lanceolate forms at times reach the size of pneumococci. Frequently, in the same cell, the nucleus contains clusters of exceedingly minute paired granules which are resolved with difficulty, while the cytoplasm contains diplobacilli of average size, together with a few of the very large lanceolate forms. Except for the constant occurrence of these different forms in cultures known not to be contaminated, one would have difficulty in believing that they were forms of a single organism. Rarely, the large forms have been seen in nuclei, but never in nuclei which are distended with organisms. The significance of the large lanceolate form is not clear. It may, as Wolbach suggested, be a developmental stage (a resting, resistant form) or its occurrence may be determined simply by nutritional conditions. In any case, it is distinctive of *D rickettsi*, and similar forms are not seen in typhus.

Long chains or apparently filamentous forms, though not seen by Wolbach in spotted fever, have been found in spotted fever tissue cultures and may be indistinguishable from the chain forms seen in typhus

The staining characteristics of *D rickettsi* are in general similar to those of *R prowazeki*. The Giemsa method (48) and the Macchiavello method (47) both give excellent results in smears. *D rickettsi* is rather more easily demonstrated in paraffin sections than *R prowazeki* and may be stained by a wider variety of methods, including the ordinary eosin-methylene blue stain (under ideal conditions in thin sections). A modification of the Macchiavello method (38) has recently been found to stain the elementary bodies of psittacosis red, against a blue cytoplasmic background. This method was found in the author's laboratory to be unsatisfactory for staining spotted fever rickettsiae. There are two modifications of the Macchiavello method that stain *D rickettsi* very sharply and deeply in sections of the guinea pig testicle. By the following modification, the organisms stain deep red and are clearly distinguishable, even though the surrounding tissue is also partly red.

- 1 Fix tissues in Regaud's fluid, cut thin paraffin sections and run through xylol and graded alcohols to distilled water in the usual way

- 2 1% aqueous methylene blue overnight

- 3 Decolorize in 95% alcohol

- 4 Counterstain in 0.25% aqueous basic fuchsin for 30 minutes

- 5 Decolorize rapidly (about 3 seconds) in 0.5% citric acid

- 6 Differentiate rapidly in absolute alcohol

- 7 Clear in xylol and mount in gum dammar

The second modification stains the rickettsiae deep blue against a background which is partly red and partly blue.

- 1 to 5 Procedures same as above

- 6 Wash lightly in distilled water

- 7 Counterstain again in 1% aqueous methylene blue for 5 seconds

- 8 Differentiate in 95% alcohol

- 9 Absolute alcohol and xylol. Mount in gum dammar

It should also be noted that *D rickettsi* may be stained fairly well by Goodpasture's method (86).

**Cultivation, and growth requirements** In general, the conditions which permit the existence and multiplication of *D rickettsi* are similar to those necessary for *R prowazeki*. The latter in the louse is confined to the cells lining the intestinal tract, while *D rickettsi* in the tick is found within practically every type of cell and in every organ. In mammalian tissues, *R prowazeki* grows only in vascular endothelium and in the serosal cells lining the peritoneal cavity (87), while *D rickettsi*, in addition to the above cell types, also grows in smooth muscle cells of arteriolar walls and in macrophages (17). This ability to utilize a wider variety of cells as hosts is of great interest, but its significance, in terms of growth requirements, cannot be learned until we know the details of the metabolic processes in the various types of cells.

*D rickettsi*, in guinea pigs, shows a preference for the scrotum and testes,

probably because of the lower temperature obtaining there, and in plasma tissue cultures grows best at 32 C. The effect of riboflavin-deficiency has not been carefully studied. Moderate numbers of spotted fever rickettsiae have been found by the author in the peritoneal cavity of riboflavin-deficient rats following intraperitoneal injection, in contrast to the absence of rickettsiae in rats similarly injected and on a normal diet, but the effect is much less striking than that seen in typhus. By producing satisfactory riboflavin deficiency in a more susceptible animal like the guinea pig, results of interest might be obtained.

In plasma tissue cultures, the restricted multiplication of *D. rickettsi* in the cytoplasm of cells and its unrestricted multiplication in nuclei (see below) should furnish a clue to its metabolic requirements. In spite of many attempts, it has been found impossible, by alterations in the temperature, pH, gaseous content, or chemical composition of the tissue culture medium, to produce marked intracytoplasmic multiplication of spotted fever rickettsiae, comparable to that seen with typhus rickettsiae (54).

In the yolk sac of the developing egg embryo (73) and in the Zinsser-Weitz-Patrick medium, the large number of spotted fever rickettsiae seen in smear preparations suggests that the organisms grow more freely than they do by other methods. It would be of interest to study cultures of the above types in paraffin sections, in order to find out exactly what types of cells are invaded and how freely the organisms grow in the nucleus and cytoplasm of the infected cells.

*Viability under various environmental conditions.* Spotted fever rickettsiae are killed in a few minutes by exposure to moist heat at 50 C or to chemical agents, and in a few hours by thorough desiccation at room temperature. Infectious guinea pig blood retains its virulence at room temperature for only about a week, and the titre of infectivity diminishes rapidly during that period. At 5 C, virulence is maintained for somewhat longer periods, ranging up to fifteen days. At -7 C, brain and spleen from infected guinea pigs remain virulent, either when suspended in glycerine or when merely sealed in containers to prevent drying, for periods ranging up to a year (88). It is probable that retention of virulence for even longer periods of time, and with slower loss of titre could be obtained at -50 C.

The evidence obtained from paraffin sections of plasma tissue cultures indicates that *D. rickettsi* survives only for brief periods when set free by the disintegration of cells (61, 54). Extracellular rickettsiae are seen in the fibrin clot at the edge of the tissue fragments in somewhat larger numbers than are typhus rickettsiae, but are never present in such concentrations as to suggest their survival for more than a few hours.

*Cytological studies.* The striking feature of *D. rickettsi* in plasma tissue cultures is its apparent preference for the nuclei of cells, where it grows in compact clusters (61, 54, 18). At times, the entire nucleus becomes distended with organisms, and there is definite peripheral condensation of the nuclear chromatin, similar to that seen in association with the intranuclear inclusions of certain virus diseases. Occasionally, from one to five . . .

may be seen within a nucleus, and these clusters show a marked tendency to maintain a spherical form. In the cytoplasm of the cells, the rickettsiae are never in clusters, but grow sparsely and are arranged diffusely.

The occurrence of these spherical intranuclear clusters, which at times are composed of such minute elements that they appear to be finely granular, is naturally suggestive of the intranuclear inclusions seen in filterable virus infections. The resemblance is made still more striking by the fact that frequently the organisms forming the intranuclear clusters are stained bright red in Giemsa preparations, while the intracytoplasmic organisms stain blue. Nicolau (89) has reported that with certain stains the intranuclear inclusions of herpes simplex may be shown to be composed of compact masses of bacillary structures, and in his illustrations the picture is remarkably similar to that of intranuclear spotted fever rickettsiae. The author (90) studied the intranuclear inclusions of Virus III in tissue cultures, fixed and stained by the Regaud-Giemsa method, and was unable to demonstrate any internal structure. Whatever the nature of the herpes inclusions may be, it is unsafe to generalize regarding the nature of intranuclear inclusions in general, since it appears probable that their nature may vary (3) and that certain types of intranuclear inclusions may be caused by chemical injury.

Multiplication of parasites within the nuclei of their host cells is a very uncommon occurrence. Excluding "virus bodies" like those of herpes, the only definite microorganisms other than *D. rickettsi* which exhibit this phenomenon are relatively large protozoa, such as *Karyophagus salamandrae* (91) which is a parasite of the salamander.

The unique intranuclear localization of *D. rickettsi* was utilized in the classification of atypical strains which gave ambiguous cross-immunity reactions (18). Alexander and Mason (92) have recently found this criterion, as applied to sections of cultures on the chorio-allantoic membrane of the chick embryo, of great value in classifying certain atypical strains of rickettsial diseases in South Africa.

*Pathology, and pathogenicity for animals.* The pathology of spotted fever is in general similar to that of typhus, the essential lesion being a specific endangitis. Focal brain lesions, formerly believed to be peculiar to typhus, have been shown by Lillie (93) to develop in spotted fever guinea pigs which do not die in the earlier stages of the disease. Previous failure to observe these lesions is probably due to the fact that both human patients and guinea pigs have died before there was time for the lesions to develop.

Monkeys, rabbits and guinea pigs are susceptible to spotted fever, and the pathological picture in these animals is essentially like that seen in man. Dogs are susceptible to infection with fièvre boutonneuse and are probably important in maintaining the disease in nature. A number of other lower animals are capable of harboring the infection, usually in a clinically inapparent form. These hosts include practically all North American rodents, and in South America the wild dog and the opossum.

The scrotal reaction in guinea pigs reacting to highly virulent strains of

spotted fever is usually of a different type from that seen in typhus. Following a brief period of simple swelling and redness, the scrotum in these strains of spotted fever becomes deep red, often with petechial hemorrhages, and eventually dark purplish-black or even gangrenous. A similar scrotal reaction often occurs in human patients. Scrotal sac exudate is not copious, as in murine typhus, but acute arteritis with thrombosis is the outstanding feature of the lesion. These changes occur in guinea pigs injected subcutaneously as well as in those injected intraperitoneally, while the typhus lesion, which is an acute inflammation of the tunica vaginalis, occurs only after intraperitoneal injection.

In virulent spotted fever, rickettsiae are usually not found, or found only in very small numbers, in smears from the tunica surface, but are seen in large numbers in the endothelium and smooth muscle tissue of arterioles beneath the surface, both in the scrotum and in the testis.

Certain milder strains of spotted fever, notably *fièvre boutonneuse* (94) and certain mild strains originating in the United States (61), give an acute exudate with moderate numbers of cells containing rickettsiae in the scrotal sac of the guinea pig, simulating the typhus reaction. Rickettsiae are present in much smaller numbers than in typhus, however, there being rarely more than twenty-five to thirty organisms per cell, and the organisms appear larger and often more lanceolate in shape. Intranuclear rickettsiae are occasionally recognized in smears from the scrotal sac. They have recently been found in considerable numbers in guinea pigs treated with sulfadiazine and sulfathiazole (95).

Certain strains of spotted fever rickettsiae cause practically 100 per cent mortality in the guinea pig, while other strains are never fatal. It is not known whether the virulence for the guinea pig corresponds accurately to the virulence for man. Mildly virulent strains (for the guinea pig) have been recovered in the western United States, as well as in many other parts of the world.

*Strain variation.* Spotted fever, like typhus, occurs in man in various parts of the world and shows considerable clinical variation. In some instances, the clinical variation depends entirely on the severity of the infection, but certain differences are due to biologically modified strains of the etiological agent.

The diseases now recognized as belonging to the spotted fever group are 1 Rocky Mountain spotted fever, 2 Eastern spotted fever, 3 *fièvre boutonneuse* (94), 4 São Paulo typhus (96, 97), and 5 South African tick-bite fever (92). These names are given here because they represent localized infections which were studied by different observers. The exact relationship between the strains causing these kinds of spotted fever is not yet entirely clear. It has, however, been established by thorough study that there are no important differences between the agents of Rocky Mountain spotted fever, Eastern spotted fever, and São Paulo typhus. (The last is more appropriately called Brazilian spotted fever.) Unless definite immunological differences are demonstrated, *Dermacentor* *rickettsi*, the name given to the rickettsia of Rocky Mountain spotted fever should be applied to the etiological agents of all three of these diseases. It should be noted, however, that the species of ticks commonly involved in transmission to man is different in the three diseases. *Dermacentor*

*andersoni* for Rocky Mountain spotted fever, *Dermacentor variabilis* for Eastern spotted fever, and *Amblyomma cajennense* for Brazilian spotted fever

Fièvre boutonneuse differs clinically from the above three diseases in the presence of a localized primary sore and an inflammatory reaction in the regional lymph nodes. Morphologically, in ticks and tissue cultures, the rickettsia of fièvre boutonneuse is identical with that of Rocky Mountain spotted fever (94). Parker (98) has shown, however, that although guinea pigs recovered from either infection are solidly immune to the other, a vaccine made from formalinized tick tissues, which protects guinea pigs effectively against Rocky Mountain spotted fever, Eastern spotted fever, or Brazilian spotted fever, is completely ineffective against fièvre boutonneuse. The author (94) has confirmed this observation with careful attention to the question of dosage. This finding is of great interest, and the underlying mechanism should be investigated further, since it involves a principle which, so far as I am aware, has not been brought to light before. Since the arthropod vector and intermediate mammalian host are different in the two diseases, the observation is reminiscent of that made by Laidlaw and Duncan in distemper, namely that, using the same strain of virus, vaccine made from formalinized ferret tissues, and effective for immunizing ferrets, was ineffective for the immunization of dogs.

This immunological difference justifies one in regarding fièvre boutonneuse as a somewhat modified strain, and the etiologic agent could, as suggested by Alexander and Mason (92), appropriately be called *Dermacentroxenus rickettsi* (var.) *conori*. The differences between the two organisms appear to be of about the same magnitude as those between *Rickettsia prowazeki* var. *prowazeki* and *R. prowazeki* var. *mooseri*.

The identity of tick-bite fever of South Africa has been the subject of considerable dispute, since Pijper (99) has maintained that it is immunologically unrelated to spotted fever. The disease is of very feeble virulence for guinea pigs, and this fact makes the interpretation of cross-immunity tests difficult, particularly in that the slight non-specific immunity caused by an unrelated infection may upset the delicate balance and give false negative results. In the author's opinion, Mason and Alexander (92) by the careful application of a number of available criteria, including the demonstration of intranuclear localization of the causative organism in chick embryo cultures, have established tick-bite fever as a member of the spotted fever group. These workers suggest the name "*Dermacentroxenus rickettsi pijperi*" for the etiological agent of the disease, but it is not clear on just what basis they would separate this organism definitely from that of fièvre boutonneuse. In general, it seems unwise to create variety names unless differences are found which can be demonstrated with regularity by laboratory methods.

**Immunology** One attack of spotted fever confers lasting immunity against subsequent attacks, and it is known that the vaccine made by Spencer and Parker against Rocky Mountain spotted fever protects also against Eastern spotted fever. Although it is ineffective against fièvre boutonneuse, it is probable that a strictly homologous vaccine made by the same method would be

successful. It would be of interest to find out whether vaccine made from *Dermacentor andersoni* ticks with fièvre boutonneuse rickettsiae would be sufficient, or whether it would be necessary to use the common tick vector of fièvre boutonneuse (*Rhipicephalus sanguineus*).

Several of the methods detailed above for obtaining high concentrations of *R. prowazeki* for vaccine purposes are applicable to spotted fever. Vaccine made from the tissues of ticks by the method of Spencer and Parker (100) is the only one used in practice at present, although Cox (73) believes that the yolk sac method is superior. It should be noted that the tick vaccine for spotted fever antedated Weigl's louse vaccine for typhus (70). Immunity produced by killed rickettsiae of any source is relative rather than absolute, and vaccination should be repeated yearly. Available evidence suggests that the value of vaccination lies partly in the reduction of severity of the infection, which is occasionally, at least, acquired in spite of vaccination.

FitzPatrick (101) has obtained high concentrations of spotted fever rickettsiae by a method similar to that of Castaneda (74).

Passive immunization in spotted fever is experimentally possible, and Topping (102) has produced a hyper-immune rabbit serum for limited clinical use. This serum has not been fully evaluated, but its value on the basis of present data appears somewhat doubtful in severe cases.

It is obvious that unexploited possibilities exist in the way of immunization with living rickettsiae from the milder strains. Tick-bite fever of South Africa, for example, is a disease with no mortality, and its rickettsiae, which have been shown to immunize guinea pigs against fièvre boutonneuse, conceivably might immunize against more virulent strains of spotted fever. Cox (73) has also reported the development of an avirulent strain of spotted fever rickettsiae by prolonged cultivation in the yolk sac. This strain causes in guinea pigs no febrile reaction or other evidence of illness, but produces solid immunity to massive doses of highly virulent rickettsial strains.

**Agglutination reactions.** The Weil-Felix reaction is usually positive in spotted fever, the characteristic finding being an increase from a low or negative reaction to a positive reaction in moderate titre with *Proteus vulgaris* OX 19 and OX K. Positive reactions at a dilution of 1:160 are occasionally seen in other diseases. Specific agglutination with rickettsial suspensions is also possible, the suspensions being made by concentrating the organisms from the yolk sac or other sources. This specific agglutination test has advantages over the Weil-Felix reaction in that it is more reliable and gives a presumptive diagnosis earlier in the course of the disease.

**Latent infection.** The infective agent of spotted fever differs from that of typhus in the fact that it disappears much more rapidly from the tissues of recovered or latently infected experimental animals (80).

**Chemotherapy.** As in typhus, Topping (83) found prontosil and sulfapyridine of no value and possibly even detrimental when administered to guinea pigs with spotted fever. The author with von Hofgaarden (103) has tried sulfathiazole, neoprontosil, sulfadiazine, fuadin, and atabrine in spotted fever guinea

pigs, and obtained negative results, similar in all instances to those reported by Topping but with even greater emphasis on the detrimental effects. In guinea pigs treated with sulfadiazine, intranuclear rickettsiae were for the first time seen in considerable numbers in smears from the scrotal sac and in sections from the testis.

**Diagnosis** Methods for the diagnosis of atypical strains of spotted fever have been described above in the discussion of strain variation and in the section on typhus fever.

#### RICKETTSIA TSUTSUGAMUCHI (TSUTSUGAMUCHI DISEASE)

**Etiological studies** Although long suspected of being a rickettsial disease, proof of the etiological relationship of a rickettsia (*R. tsutsugamuchi*) (*R. nipponica*) (*R. orientalis*) to the disease was obtained much later than in the case of typhus and spotted fever. One reason for this is the low susceptibility of experimental animals. In 1928, Ishiwara and Ogata (104) transmitted the disease to the rabbit by intratesticular inoculation and demonstrated the rickettsiae in the interstitial cells of the testis. Nagayo and his co-workers (105) showed that intraocular injection in the rabbit resulted in an ophthalmitis, and demonstrated rickettsiae very clearly in the endothelial cells overlying Descemet's membrane.

Proof of the etiological relationship of the organism to the disease is based on its constant demonstration in cells of the above types in rabbits after injection with material from human patients, and also on the cultivation of the organism in tissue culture for long periods of time (106) with full retention of its virulence when introduced into rabbits by the above methods. Although obviously transmitted by the larval stage of mites (*Trombicula akamushi* and *T. deliensis*), no studies of the location and morphology of the organism in mites have been published.

The disease has been less completely studied than typhus and spotted fever, cytological studies being particularly incomplete. For this reason, its exact relationship to the latter two diseases was for a time obscure. Lewthwaite and Savor (107) have, however, demonstrated rather conclusively that tsutsugamuchi rickettsiae are immunologically distinct from those of typhus and spotted fever. An important factor in their work was the fortunate chance of transmitting the disease to guinea pigs with resistance lowered as a result of a vitamin-deficient diet (108), thus gaining the opportunity of carrying out cross-immunity tests with the other rickettsial diseases under approximately similar conditions.

**Size, morphology, and staining** *R. tsutsugamuchi* shows considerable morphological similarity to certain forms of *R. prowazeki* and *D. rickettsi*. The organism is a short diplobacillus with bipolar staining. Lewthwaite and Savor (108) give the average limits of size as 0.8 to 2.0  $\mu$  in length and 0.3 to 0.5  $\mu$  in width. The organism tends to be shorter and plumper than typhus and spotted fever rickettsiae, and it is believed that differential diagnosis could be made on morpho-

logical grounds alone by an experienced observer Ogata and Unno (109) depict short chains The staining reactions are like those of the other rickettsiae, but bipolar staining is perhaps more prominent The organisms have not been described as capable of distending cells, and are usually diffusely scattered rather than clustered

*Cultivation, and growth requirements* Observations in experimental animals and in tissue culture (106) suggest that *R tsutsugamuchi* is an obligate intracellular parasite, and that its growth requirements are essentially like those of the rickettsiae of typhus and spotted fever The organism grows freely in the cytoplasm of the interstitial cells of the rabbit testis and the endothelial cells overlying Descemet's membrane No convincing evidence of intranuclear localization has been presented

*Pathology, and pathogenicity for animals* Pathological studies in man have not been reported From the fact that the cutaneous rash resembles that of typhus and spotted fever, one might conclude that the pathology is probably an endangitis, as in the latter diseases Lewthwaite and Savor (108) described focal brain lesions in guinea pigs similar to those seen in typhus and spotted fever The occurrence of ascites in infected guinea pigs, with rickettsiae in cells of the ascitic fluid, was constant in the disease described by these workers Susceptible animals, in addition to man, are monkeys, rabbits, rats, and guinea pigs, but the disease is very difficult to establish and maintain in these animals (excepting the rabbit following injection by the routes mentioned above) The vole is apparently the reservoir of the disease in Japan, while in Formosa the rat is the reservoir Although Lewthwaite and Savor (108) established and maintained a strain in scorbutic guinea pigs, they were unable to repeat this achievement Progress would be more rapid if an animal highly susceptible to the disease by the ordinary routes of inoculation could be found

*Strain variation* Clinically the disease occurs in a mild and a severe form The severe form is characterized by a necrotic local lesion at the site of attachment of the vector and inflammation of the regional lymph nodes, while these lesions are absent in the mild form It is of interest to note that this situation is the reverse of that obtaining in the spotted fever group, where the mild forms (fièvre boutonneuse and tick-bite fever) are the ones associated with a severe local reaction No criteria for the recognition of true variant strains of *R tsutsugamuchi* have been described It is clear that tropical typhus of the rural type (rural typhus) is essentially identical with tsutsugamuchi disease

*Immunology* The immunity conferred by an attack of tsutsugamuchi disease is apparently less complete than in typhus and spotted fever Reinfection is fairly common, but second attacks are usually mild Vaccines could undoubtedly be prepared by the methods detailed above, since the organism grows in tissue culture, and would probably grow in the yolk sac and in other media containing living cells

The Weil-Felix reaction, which is important in diagnosis, is characterized by agglutination of *Proteus vulgaris* OX K in high serum titre

## RICKETTSIA RUMINANTIIUM (HEARTWATER)

*Etiological studies* Heartwater, a highly fatal and economically important disease of cattle, sheep, and goats, was etiologically obscure until Cowdry (110) demonstrated in 1926 the causative agent, *R. ruminantium*, in the endothelial cells of the kidney and brain of sheep dying of the disease. By studies in the tick, *Amblyomma hebraeum*, Cowdry (111) soon demonstrated the inseparability of the organism from infectivity, and established the etiological relationship of the organism to the disease by methods similar to those applied in typhus and spotted fever. The infection is not hereditary in the tick, but is transmitted from the nymph to the larva and from the larva to the adult.

*Size, morphology and staining* Morphologically, *R. ruminantium* is rather strikingly different from typhus and spotted fever rickettsiae. Rod-shaped forms are infrequent, and most of the organisms are rounded or elliptical, with occasional horseshoe shapes resembling certain forms assumed by *Bartonella canis*. The organisms are confined to the cytoplasm, and occur there in large numbers. They are at times rather diffusely arranged, but often in definite clusters like those of the elementary bodies of psittacosis. The range in size is approximately that of *D. rickettsi*. The organism is not filterable.

On morphological and other grounds, it would perhaps be preferable, on the basis of present knowledge, to assign this organism to a different genus in the family RICKETTSIACEAE, but to do so would serve no useful purpose at the present time.

*Cultivation, and growth requirements* No reports are available on the cultivation *in vitro* of *R. ruminantium* by any of the methods involving the use of living or surviving cells, and the organism refuses to grow in cell-free media. In ticks and in mammalian tissues, it behaves as an obligate intracellular parasite. Virulence of infected tissues at room temperature is retained for only a few hours.

*Pathology, and pathogenicity for animals* Pathologically, *R. ruminantium* appears to resemble the rickettsiae of typhus and spotted fever in that it infects primarily the vascular endothelium. A striking difference from the above diseases is the accumulation of large quantities of fluid in the pleura, peritoneum, and pericardium (whence the name heartwater). The liver, spleen, and kidneys are congested. Microscopically, there are slight perivascular accumulations of inflammatory cells, but no thromboses. The rickettsiae are seen in sections in the endothelium of small blood vessels, particularly in the brain and kidneys, and can be most readily demonstrated in smears made by scraping the endothelium of large blood vessels.

Small laboratory animals are said not to be susceptible (Alexander, Mason, and Neitz (92), although Balozet (112) claims to have carried the infection for several generations in guinea pigs, rabbits, and rats.

*Immunology* In recovered animals, even after 105 days, rickettsiae are still demonstrable in the vascular endothelium (113) and the disease may be transmitted from sheep to sheep by scrapings of the endothelium at a time when the blood is no longer infective. The immunity appears to be that of tolerance,

and since immunity is only gained by active infection, no satisfactory method of vaccination has been devised. Immune serum treatment has likewise been found of no avail, and inoculation with attenuated virus has given no results of practical value. It is said that immunity may be broken down by inoculation with a different strain of the disease (113).

#### RICKETTSIA WOLHYNICA (TRENCH FEVER)

*Etiological studies* The role of the body louse in transmitting trench fever in man was established during the world war of 1914-1918 by British and American Commissions (114, 115). The disease was readily transmitted to human volunteers by rubbing the feces of infected lice into the scarified skin and also by allowing infected lice to feed on the volunteers. In 1916, Töpfer (8, 9) described extracellular rickettsiae in the intestines of lice, and suggested that these organisms might be the cause of the disease. Similar extracellular organisms were described by Munk and da Rocha-Lima (10) in presumably normal lice and named *Rickettsia pediculi*.

Arkwright, Bacot, and Duncan (116) showed that lice proven non-infective and free from rickettsiae regularly became infective about nine days after feeding on patients with trench fever, and that extracellular rickettsiae regularly appeared in the intestinal tract and feces of such infective lice. While Bacot was studying typhus fever in Poland, he acquired trench fever (7). Stock lice, entirely free from microorganisms of any sort, were being fed on Bacot at the time. Twenty days after the onset of his illness, extracellular rickettsiae appeared in these lice and also in other lots of stock lice fed on him at the time.

Evidence of the etiological relationship of the organism *Rickettsia wolhynica* (also called *R. quintana*) to trench fever is thus of an incomplete nature, and it can only be said that the organism is probably the cause of trench fever. The failure to transmit the infection to experimental animals and the practically complete disappearance of the disease following the war of 1914-1918 have made further progress impossible. Recently, the disease has been reported (117) in individuals on whom presumably normal lice were being fed in the process of making typhus vaccine by the Weigl method.

*Morphology and staining* *R. wolhynica* is somewhat more deeply stained in film preparations than the more characteristic rickettsiae. The organisms are also somewhat plumper, more definitely oval, and less pleomorphic than typhus rickettsiae. It would, however, be difficult to distinguish *R. wolhynica* from *R. prowazeki* on morphological grounds alone.

*Growth requirements* *R. wolhynica* has not been cultivated in cell-free media. It is more resistant to heat and drying than *R. prowazeki*, resisting dry heat at 80 C for twenty minutes and desiccation in sunlight for four months. In the intestinal tract of lice, the organism is concentrated along the borders of the lining cells, suggesting that it may depend for its growth on some product of the metabolism of these cells. Modern methods of cultivation in media containing living cells, such as those detailed above, have never been applied to

the study of *R. wolhynica*, and it seems probable that much information would be gained by the use of these methods

*Immunology* The disease is characterized by relapses which may occur as late as two years after onset. Immunity is slow in development and is probably the immunity of tolerance, since recovered cases continue to infect lice, previously free from organisms, for many months

#### RICKETTSIA BURNETI (DIAPORICA) (Q-FEVER)

*Etiological studies* In 1937, Burnet and Freeman (118), in studying an epidemic among individuals working in slaughter houses or on dairy farms in Australia, were able to transmit the disease to experimental animals, and they described a rickettsia-like organism in smears from the spleens of infected mice. This organism was named *Rickettsia burneti* by Derrick (119). A similar organism was recovered by Davis and Cox (120) from ticks (*Dermacentor andersoni*) collected in Montana. This organism was found to be pathogenic for guinea pigs, and its properties were carefully studied before there was any indication that it might be related to human illness. It was called *Rickettsia diaporica*. Dyer (121) reported a case of accidental laboratory infection with *R. diaporica*, and showed that there was cross-immunity between Australian Q-fever and the Montana infection in guinea pigs (122). This observation has been confirmed and careful comparison of the two organisms, *R. burneti* and *R. diaporica*, has shown that they are closely related if not identical. Minor differences in behavior in guinea pigs, not unlike the differences described in various strains of spotted fever, have been observed, but no differences inconsistent with the fundamental similarity of the two organisms. The etiological relationship of *R. burneti* and *R. diaporica* to Q-fever in Australia and in the United States, respectively, has been established by guinea pig inoculation and serological tests. There have been several cases of laboratory infection, both in Australia and in the United States, and recently an institutional outbreak occurred in Washington, D. C. (123).

*Filterability, morphology, and staining* Workers in both countries have studied the biological properties of the organisms and found them to be filterable through Berkefeld filter which apparently did not allow typhus and spotted fever rickettsiae to pass (120). This observation is the basis for the specific name "diaporica" given by the American investigators. Uncertainty exists regarding the occurrence of the organisms in an invisible form.

Morphologically the rickettsiae of Q-fever are similar to those of spotted fever and typhus, but appear somewhat larger (perhaps because they stain more deeply). The organisms, in infected animal tissues, apparently grow both intracellularly and extracellularly. Within infected cells, which are chiefly mesothelial and reticulo-endothelial cells, the organisms tend to form compact spherical clusters, the pattern of infection being quite similar to that of *Bartonella bacilliformis*.

*Cultivation, and growth requirements* Growth occurs, both intracellularly and extracellularly, in the various media containing living cells commonly used for

the growth of rickettsiae and filterable viruses (124) Multiplication has not occurred in any cell-free medium Particularly massive growth is obtained in the yolk sac of the developing chick embryo The culture requirements of *R. diaporica* appear to be similar to those of *Bartonella bacilliformis*, but no multiplication occurs in cell-free media which have been successfully used for the latter organism The same can be said for *Bartonella muris*, however, which closely resembles *B. bacilliformis*, and it seems probable that *R. diaporica* will eventually be cultivated in a cell-free medium Careful cytological studies in tissue cultures have not been carried out

*Pathology, and Pathogenicity for animals* In a fatal human case reported by Lillie, Perrin, and Armstrong (125), the gross pathological findings were pulmonary edema and congestion, firm granular consolidation of the upper lobe of the right lung posteriorly, and a large soft spleen The other organs showed no important changes Microscopically, the picture in the lung was that of an atypical pneumonia, with much fibrin in the alveoli and bronchioles, and in general a scanty mononuclear cell reaction instead of the purulent reaction seen in typical bacterial pneumonia Similar pathological changes were seen in the lungs of experimentally infected monkeys In neither the human nor the monkey lungs were rickettsiae demonstrated histologically The similarity of the pathologic picture to that described in fatal cases of atypical pneumonia of unknown origin by Longcope (126) and by Kneeland and Smetana (127) was striking The picture is also rather similar to that of certain cases of psittacosis pneumonia (128)

In guinea pigs, after an incubation period of two to eighteen days depending on the amount of infective material injected, fever follows for four to six days, and loss of weight and appetite Guinea pigs have been successfully infected by the inoculation of human blood or urine There is practically no mortality in the guinea pig, but at necropsy a large soft spleen is found and rickettsiae may be seen, both free and in mononuclear cells, in smears of the spleen stained by the Giemsa or the Machiavello method

In the guinea pig, Lillie (129) has described perivascular accumulations of lymphocytes and rare focal brain lesions like those seen in typhus and spotted fever Granulomatous tubercle-like lesions, with giant cells, are also described in the spleen, liver, and other organs

In mice, Burnet and Freeman (118) have described small, necrotic foci in the liver, and the presence of rickettsiae in the Kupffer cells of that organ

*Immunology* Vaccines prepared from yolk sac suspensions or from infected mouse spleens (130) are effective in building up active immunity Hyperimmune serum also gives considerable protection In general, the immunological aspects of the disease appear to be similar to those in typhus and spotted fever

#### RICKETTSIA CANIS AND RELATED ORGANISMS

*Rickettsia ovina* was observed in sheep in 1930 by Lestoquard and Donatien (131) Later, a similar organism (*R. canis*) was reported in dogs (132),

and a third variety (*R. bovis*) in cattle (133) These three organisms are morphologically identical They all produce febrile illness in their mammalian hosts and have in common the fact that they parasitize the monocytes of the circulating blood Evidence suggesting tick transmission has been presented in the case of all three organisms, but morphological studies of the organisms in their probable vectors have not been reported

*Rickettsia ovina* was first found in monocytes of the circulating blood from a sheep suffering from a febrile illness after inoculation with emulsions of organs of the tick, *Rhipicephalus bursa* A second sheep in which the rickettsiae were seen had a concomitant infection with *Babesiella ovis* A third instance of infection with *R. ovina* was encountered in a splenectomized sheep inoculated with blood from a second splenectomized sheep in which a double infection with *Babesiella ovis* and *Anaplasma ovis* was present *Rickettsia ovina* has been very incompletely studied, and it is impossible to draw any conclusions regarding its exact nature

*Rickettsia bovis*, similarly, was found in the circulating monocytes of a bull on which ticks of the genus *Hyalomma* had been fed The ticks were infected with *Theileria dispar* and had transmitted this organism to the bull The infection with *R. bovis* was presumably derived from the ticks

*Rickettsia canis* has been studied in somewhat greater detail It was first found in the circulating blood of dogs suffering from a fatal illness which began a few days after many ticks (*Rhipicephalus sanguineus*) had fed upon them Later the infection was found to be common in dogs and to occur in both an acute and a chronic form Ticks fed on dogs suffering from the infection were shown to be infectious when ground up and injected into other dogs In one instance, infection was produced in a monkey by the inoculation of ground-up larvae issuing from a female *Rhipicephalus sanguineus* which had engorged on a dog suffering from acute infection with *Rickettsia canis*

Morphologically, *R. canis*, like the other two members of the group "rickettsiae of monocytes," occurs usually in compact intracytoplasmic aggregations or groups, often indenting the nucleus but never invading it Several clusters may occur in a cell, the pattern produced simulating that of *Bartonella bacilliformis*, *Rickettsia diaporica* and the organism of psittacosis The individual elements are 0.2 to 0.3  $\mu$  in diameter when closely packed, but range up to 0.5 or 0.6  $\mu$  in diameter when separated from one another They are rounded, elliptical, polygonal or rarely coccobacillary or bacillary The staining reactions are similar to those of the typical rickettsiae, except that the structures do not stain by Castaneda's method Filtration experiments gave negative results

The infection is transmissible to dogs and monkeys by intravenous or subcutaneous injection of infected blood or emulsions of lung, spleen, or brain tissue Dogs which recover from the infection remain chronically and latently infected, and show an immunity of tolerance, similar to that seen in infection with *R. ruminantium* Relapses can be brought about by intercurrent infection or by splenectomy

*R. canis* appears to be distinct from bartonella, eperythrozoon, hepatozoon,

and piroplasma, and is obviously unrelated to the rickettsia of fièvre boutonneuse. In view of the fact that *R. canis*, *R. ovina* and *R. bovis* are primarily parasites of the circulating monocytes, it is the author's opinion that more detailed studies must be carried out before a decision can be reached as to whether these organisms should be grouped with the rickettsiae. If they should be so classed, they should be given generic names other than Rickettsia. These organisms have been discussed here largely for the sake of completeness. A detailed account of them may be found in a paper by Donatien and Lestogard (113).

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#### CORRECTIONS

Vol 5 page 250, table 3, column 4, line 10 "193" should read "141"

Vol 6, page 25, lines 5 and 6 should read " as Hadley (79) questions whether the mucoid-rough cultures of Edwards were *pure-phase* cultures"



# ELEMENTARY BODIES OF VACCINIA

J E SMADEL AND C L HOAGLAND

*The Hospital of the Rockefeller Institute for Medical Research, New York*

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Vaccine virus has been studied so diligently and successfully during the past decade that it now stands as the best-defined member of the group of animal viruses. This recent development of knowledge represents the flowering of a field carefully cultivated for half a century and sporadically tended for the preceding hundred years. A number of factors have contributed to the sustained interest in the study of the nature of the virus of vaccinia, namely, the importance of cowpox in prevention of one of the great scourges of mankind, the early recognition of this agent as a member of the group of viruses and the tendency to regard it as one of the classical examples of the group, and, finally, the ease with which this virus is handled in the laboratory. The present review will trace the development of methods which provide highly purified preparations of virus. It will deal with the evidence which indicates that the elementary body represents the infective unit of the virus. Furthermore, data bearing on the physical, chemical and antigenic structure of the elementary bodies will be presented and discussed as regards the light they focus on the position of vaccine virus in the scale of infectious agents. A number of earlier reviews may be consulted for a discussion of other aspects of the subject of vaccinia-variola (6, 25, 27, 122).

## DEVELOPMENT OF METHODS FOR THE PREPARATION OF HIGHLY PURIFIED VIRUS

The aura of mystery which has surrounded the subject of viruses for so many years is being gradually dispersed. It has receded step by step with the development of methods that have made available for study virus preparations of increasing degrees of purity. Knowledge gained from the examination of such preparations has replaced ignorance and speculation, as a result, viruses are no longer regarded as noxious infectious agents which lie entirely apart from bacteria, protozoa and animal cells.

During the era when microscopy and morphological structure dominated experimental medicine and when Laveran's description of the malarial parasite was new, workers observed many bizarre forms in cells infected with the virus of vaccinia and in vaccine lymph (6). Only two of these are now considered significant, they are the cytoplasmic inclusions of Guarnieri (31) and the minute particles or elementary bodies of Paschen (77). Attention has been called (28) recently to Buist's description of elementary bodies of vaccinia almost 20 years

prior to that of Paschen. It seems useless to discuss the chronology of these microscopic observations, however, for neither report was widely accredited in its day nor would either be accepted now as proving the relationship between the elementary body and the virus. This is particularly true in view of the recent work on cell particles derived from suspensions of normal tissue (10). It should be mentioned that Paschen subsequently sought for and obtained evidence (68, 78) of other types which strengthened his early hypothesis concerning the infectious nature of the elementary body.

The obligate parasitism of viruses, *z e*, their inability to multiply in the absence of living cells, long delayed the direct study of these agents, for it was obvious to all that the major portion of even the most highly infectious tissue suspensions was nonviral cellular material. The first serious attempt to obtain suspensions of elementary bodies of vaccinia free of extraneous matter was made in 1922 by MacCallum and Oppenheimer (58). These workers applied the technique of differential centrifugation to vaccine lymph obtained from infected dermal pulp of calves. Although repetition of their experiments was not reported from other laboratories, their idea for centrifugal separation of particles of a given size and density from a mixture consisting of many different kinds has been widely accepted.

Ledingham's contribution (52), nine years later, provided the background for the modern method of obtaining purified suspensions of vaccine virus. He suspended triturated material from early cutaneous lesions of rabbits in distilled water and added ether to the suspension. Following preliminary centrifugation which separated large particles and floating material from the opalescent aqueous suspension, the virus was sedimented from the water by centrifugation for 1 hour at 9000 r p m. The sediment was then resuspended in saline solution and freed of aggregates by fractional centrifugation. Craigie (12, 14) altered this technique in a number of important respects: a strain of vaccine virus well adapted for multiplication on the skin of rabbits was employed, large areas of closely clipped, unpigmented skin of healthy rabbits were gently scratched with the edge of a pad of fine wire while a highly infectious suspension of virus was applied to the site, dermal pulp was harvested 3 days later by gently scraping with a dull scalpel the inoculated surface which had been moistened with buffered water, dermal pulp diluted appropriately with buffered water was then subjected to a process of differential centrifugation similar to Ledingham's, except that the Swedish angle machine was used for sedimenting the virus, the material originally thrown down in the angle centrifuge (3500 r p m for 1 hour in flat tubes) was washed several times by resuspending in dilute buffer solution and recentrifuging, and finally, aggregated particles were removed from the suspensions of washed virus by prolonged centrifugation in the International machine.

It is worthwhile to discuss certain fundamental principles which are embodied in Craigie's technique for it is by the application of these principles that one may reasonably expect to obtain pure preparations of other animal viruses. One of these is the simultaneous inoculation with active virus of myriads of cells without damage to the underlying tissue and probably without appreciable

damage to most of the inoculated cells themselves. The virus multiplies at essentially the same rate in all of the inoculated cells, hence, when harvested at an appropriate time, each cell yields a maximum amount of virus. Furthermore, at this time comparatively few cells have undergone destruction, therefore, a minimal amount of cellular debris contaminates the preparation. Another fundamental point concerns collection of material only from infected cells. This is accomplished by covering the blanket of virus-laden surface cells with a hypotonic solution and then applying a gentle shearing force exerted with a blunt scalpel. Under these conditions the diseased cells apparently rupture and discharge their contents in much the same way as do ripe grapes when squeezed between the fingers. In order to emphasize the superficial nature of the harvesting procedure, it may be mentioned that histological sections of rabbit skin taken before and after collection of dermal pulp are almost indistinguishable. Diluents with a pH slightly above 7 and with low concentration of electrolytes are essential in work of this type for the elementary bodies of vaccinia, like many other small particles, aggregate in menstrua which are below pH 7 or which contain appreciable quantities of salts.

Our modifications (30, 36, 102) of the original techniques of Craigie have been made in order to embrace the above principles more closely or to effect saving of time without compromising the purity of preparation. On the other hand, some workers (60, 63), in an attempt to increase the total yield of elementary bodies, have salvaged virus-laden materials which are ordinarily discarded. While such preparations are suitable for certain types of experimentation, they are not desirable for use in studies which demand pure virus. In a like manner, our suspensions of virus prepared from ground, infected chorio-allantoic membranes by a combined process of differential centrifugation and tryptic digestion (104) which was employed to rid the suspensions of some of the normal proteins, are useful for certain work, but these preparations do not approach the state of purity attained in the standard procedure.

#### IDENTIFICATION OF THE ELEMENTARY BODY WITH THE INFECTIVE UNIT OF VACCINIA

It can now be stated with assurance that the elementary body of vaccinia is the infective unit of the virus. Early attempts to associate elementary bodies with infectivity were essentially qualitative in character. Even as late as 1932 the important observations of Eagles and Ledingham (22) which dealt with the correlation of elementary bodies with infectivity of virus filtrates were basically all-or-none experiments. Following the development of methods for obtaining highly purified suspensions of virus and, of equal importance, the development of accurate methods for estimating the endpoint in titrations of virus suspensions (85), quantitative correlation of elementary bodies and infective units was undertaken. Needless to say, such quantitative studies make extensive use of statistical analyses and of results obtained for the most part by indirect measurements. Therefore, it is not surprising that workers in this field have frequently disagreed on the validity of methods and on the significance of observations. In order to

simplify the present discussion most of the theoretical aspects of the problem will be avoided, for those who are interested a number of the original papers (7, 32, 70, 71, 74, 107, 108, 109, 110) may be consulted. The work in this segment of the field has progressed along two converging lines, first, the estimation of the number of active virus particles needed to produce a lesion, and second, the number of elementary bodies in a single infective dose.

It must be realized that the production of a detectable vaccinal lesion in the skin of a rabbit depends on a number of factors other than the virus itself. For example, Sprunt and his co-workers have demonstrated that changes in cellular resistance of non-immune rabbits, which result from variations in the endocrine (109) and nutritional balance (108) of the animals, affect the endpoint of a given suspension of virus titrated in these animals. Furthermore, the volume of the inoculum and the number of cells exposed to the inoculum likewise affect the titration endpoints (107). While these factors are of importance in studies on the number of particles of active virus needed to produce a lesion, they are, nevertheless, controllable factors and once recognized they can be avoided.

A satisfactory method for estimating the number of infective units in a suspension of vaccine virus was introduced by Parker and Rivers (74), and, with some modification it is now used almost exclusively for accurate work. Multiple intracutaneous inoculations are made in rabbits with each of a series of dilutions of virus material. The range of dilutions is chosen so that the most concentrated suspension produces lesions in a high percentage of inoculated sites, while the least concentrated suspension produces few if any lesions when injected. The 50 per cent endpoint is then calculated by the statistical method of Muench (85). The numerical value thus obtained represents the logarithm of the dilution of virus suspension which produces lesions in half of the inoculated areas. Titration endpoints obtained by this method are reproducible within  $10^{0.1}$  (one-tenth of a Briggsian logarithmic unit) provided sufficient sites of inoculation are employed to give statistically significant results (36, 109, 110).

Parker (70) tested experimentally the hypothesis that a single infective particle of vaccine virus could produce a lesion. He applied a graphic method of statistical analysis to results obtained on multiple titrations of twofold dilutions of virus suspension, the dilutions were known to be in the region of the 50 per cent endpoint titer of the material. Parker was able to show that the data were consistent with such an hypothesis. Furthermore, the data failed to provide satisfactory statistical correlation with curves constructed on the assumption that two or more active particles were needed for infection. Haldane (32) applied an algebraic method of statistical analysis to Parker's data and pointed out that certain of the latter worker's computations required minor corrections, these corrections, however, did not invalidate the conclusion that one active particle could produce a lesion. It should be emphasized that experimental results of this type are obtained only when a strain of virus is employed which is highly virulent for the inoculated tissue. For example, simultaneous titrations of the CL strain of virus in the skins of rabbits and the brains of mice show a difference in endpoints of about  $10^5$  (71). It is thus apparent

that many infectious particles of this strain adapted to the skin of rabbits are needed to kill a mouse. Although it is helpful to know that under proper conditions a single infective particle of vaccine virus can produce a lesion (70, 71, 109, 110) such information does not permit one immediately to conclude that a single elementary body can initiate infection. The biological deductions concerning a single infective particle make no assumption regarding the structural nature of that particle, they are equally valid if the virus is as small as an albumin molecule or as large as a bacterium.

An estimation of the number of elementary bodies which constitute an infective unit of virus is fundamentally the same problem as the determination of the number of pneumococci needed to kill a mouse. The technical difficulties involved in enumerating elementary bodies are greater, however, than those encountered in counting bacteria. Certain of these difficulties are presented in the report of Parker and Rivers (74). These workers counted the particles in suspensions of washed elementary bodies by placing the materials in a counting chamber and examining them by ordinary dark-field microscopy. The value thus obtained was compared with the number of infective units in the same suspensions. Although a direct correlation was found to exist between the number of elementary bodies and infective units, the authors concluded that the data did not justify extrapolating the curve to determine the number of elementary bodies in a single infective unit.

Smadel, Rivers and Pickels (102) employed certain of their own data on size and density of elementary bodies, which had previously been obtained in ultracentrifugation studies (79, 98), to calculate the weight of a single dehydrated elementary body and found it to be  $5.34 \times 10^{-15}$  grams. Seven consecutive pools of washed elementary bodies were accurately titered for infectivity and then dried from the frozen state and weighed, their weights varied between 130 and 263 mg. The total number of infective units in each pool was calculated by multiplying the number of infective units per milliliter by the volume of the pool before drying. The total number of elementary bodies in each pool was estimated by dividing the weight of the dried material, expressed in fractions of a gram, by the figure  $5.34 \times 10^{-15}$  (the calculated weight in grams of a single dry elementary body). The ratios of elementary bodies to infective units determined for the seven preparations varied between 2.4:1 and 9.2:1, the average was 4.2:1.

This correlation between elementary bodies and infective units is surprisingly close, for there are a number of factors other than errors which may have been introduced in the titrations of infectivity and in the calculation of the weight of a single elementary body which could affect the ratios (102). For example, if any elementary body in the pooled suspensions were not infectious or not in a monodispersed state, or, if all of the dried material did not consist of elementary bodies, then the estimated number of virus particles per infective unit would increase. The effects produced by inactivation and aggregation of virus on the EB:IU ratio are illustrated by an experiment in which the infectivity of pooled suspensions of virus was determined before drying and after careful rehydration.

and resuspension (36) The number of elementary bodies per infective unit estimated from the titrations made before drying was 37.1, but when data from the second titration were employed, the ratio was 241.1 If one had only the latter ratio it would be impossible to guess which of the three factors was operating, moreover, it is obvious that one would not be justified in drawing conclusions regarding the number of active bodies needed to produce a lesion or regarding the purity of the preparation On the other hand, if the ratios approach unity one is certain that a high percentage of the elementary bodies is infectious and that the preparations of virus are of a reasonable degree of purity (102)

One may conclude from the experiments discussed in this section that under the proper conditions a single active elementary body can initiate infection

#### PURITY OF PREPARATIONS OF ELEMENTARY BODIES OF VACCINIA

In a sense most of the work discussed above and much of that which follows deals directly or indirectly with the purity of the preparations of virus It is appropriate to marshal certain of these facts now, in order to establish the fact that physical, chemical and immunological data obtained on purified preparations of virus represent characteristics of the elementary bodies themselves and not attributes of extraneous tissue material which is present as a contaminant

The usual criteria employed in chemistry have been well satisfied (36) Crude starting material, dermal pulp, amounts to several grams per rabbit while the final preparation, which contains almost all of the infectivity originally present, weighs only 8 to 10 mg The analytical chemical data on different lots of purified virus are remarkably constant On the other hand, phosphorus, total lipid and neutral fat decrease progressively as materials are discarded during the process of purification and are lowest in amount in the final preparations of virus Conversely, total nitrogen, phospholipid, reducing sugars (36) and copper (40) increase progressively and are present in largest proportion in the final product

Certain physical methods commonly employed for establishing the homogeneity of materials have been applied to vaccinia Suspensions of elementary bodies examined under the proper conditions show a single boundary in the analytical centrifuge (79) and in the electrophoresis apparatus (62, 93) Two other methods frequently employed for this purpose, *i e* determination of solubility and crystallization, are not applicable to the virus of vaccinia

It may be pointed out that the fulfillment of no single one of these criteria provides adequate proof of purity For example, the cholesterol content of virus prepared according to routine procedures is constant at about 2 per cent, however, cholesterol apparently is not an essential constituent of active virus for it can be removed by ether extraction without reducing the infectivity of the preparation (36) The technique of differential centrifugation employed in the purification of virus selects particles with approximately the same rate of sedimentation, therefore it is not surprising that the final preparation displays a single boundary in the analytical centrifuge Indeed it has been demonstrated experimentally (99) that equal mixtures of suspensions of washed virus and of collodion particles, graded by the same technique as is used for the virus,

sediment with a single boundary in the analytical centrifuge. A single boundary in the electrophoresis cell merely means that all the units of the material have similar electrical charge distributions on their surfaces. Here again it has been possible to show that a mixture consisting of a suspension of graded collodion particles coated with the soluble antigen of vaccinia and a suspension of elementary bodies move as a single boundary in the electrophoresis apparatus (99). Do such deficiencies in the chemical and physical methods mean that evidence of homogeneity obtained by these techniques is valueless? Of course not. It is apparent, however, that each type of datum may be misleading at times in regard to the purity of a biological preparation (81, 92).

Until recently evidence of morphological homogeneity of vaccine preparations was obtained by means of ordinary microscopic techniques (54, 121) or by ultra-violet light photography (23). Although such examinations showed that the material contained particles of uniform shape and size, these observations were subject to the same objections as the ultracentrifugal data, since neither the resolving power of the microscope nor the staining characteristics of the particles enabled one to differentiate with certainty the virus from normal tissue particles (53). Electron microscopy has just revealed a characteristic external shape and internal structure of the elementary bodies of vaccinia (30), this morphological evidence of the homogeneity of particles in suspensions of vaccine virus now offers the best single criterion of purity so far obtained for these preparations.

It may be concluded, therefore, from the evidence just summarized and from the data on elementary body infective unit ratios presented in the previous section, that the virus of *Vaccinia* can be obtained in a highly purified state.

#### PHYSICAL PROPERTIES OF THE VIRUS OF VACCINIA

Evidence substantiating the opinion that the elementary body is the virus of vaccinia has been presented in previous sections. Therefore, in discussing the physical properties of the virus one may draw on two sources of information, the earlier data obtained on crude suspensions of virus, and more recent data derived from studies on purified suspensions of elementary bodies. Information of the latter sort will be discussed in preference to the former, for not only are these data obtainable on cleaner preparations but also methods of direct optical measurement may be applied to the suspensions of elementary bodies, whereas infective titers must be used to follow the course of the virus in crude preparations.

Experiments which provide information on the size, density and morphological structure of elementary bodies will be summarized together with data bearing on the nature of the surface of the particle and of the response of the virus to osmotic influences.

*Size* Suspensions of elementary bodies when sedimented in the analytical centrifuge form a characteristic primary boundary which consistently shows a spread of about 14 per cent (79). The sedimentation constant of the slowest moving particles in the boundary is  $49.1 \times 10^{-11}$  cm/sec/dyne [4910 Svedberg units (88)]. In other experiments elementary bodies were found to have a

density of 1.16 (98) and a brick-like shape (30). These three sets of data enabled Pickels and Smadel (79) to calculate with considerable accuracy the size of elementary bodies. The average diameter of the smallest elementary bodies present in appreciable amounts is 236 m $\mu$  and, if the boundary spread is due to differences in particle size, the largest single elementary bodies are approximately 252 m $\mu$  in diameter.

Estimations of the size of vaccinia have been made from data obtained by other methods (see references, 79), and considering the technical difficulties involved, the various values are in reasonably close agreement. Direct measurements of electron micrographs of elementary bodies give values slightly lower than those estimated from the centrifugal data (30). This is to be expected since dehydrated particles are examined in the electron microscope and hydrated ones in the analytical centrifuge, the effective diameter of elementary bodies is reduced about 14 per cent by extraction of readily removable water (98).

*Density* Although it had been previously suspected that the density of small biologically active units such as viruses might be altered by varying the osmotic pressure of the suspending media, this fact was demonstrated by Smadel, Pickels and Shedlovsky (98) working with vaccinia in the analytical centrifuge. The sedimentation rates of particles which are unaffected by the medium decrease proportionately as the density of the medium is raised, and ultimately, when the densities of particles and medium are equal, no sedimentation occurs. Experiments with elementary bodies show that the decline in sedimentation rate observed in media of increasing specific gravity is not directly proportional to the density of the solution, therefore some change in the bodies must occur. The density of elementary bodies suspended in dilute buffer solutions is estimated to be 1.16 g per ml. Higher values are found if the virus is suspended in concentrated solutions of sucrose, glycerol or urea (98). In a 53 per cent solution of sucrose elementary bodies fail to sediment, hence their density under these conditions is equal to that of the medium, i.e. 1.25 g per ml. McFarlane, Macfarlane, Amies and Eagles (63) applied direct pycnometric measurements to dried elementary bodies prepared somewhat differently from ours and concluded that the dry density of the bodies is 1.26.

The density of elementary bodies suspended in simple aqueous media is, therefore, intermediate between that of bacteria 1.10 (89), and of protein 1.33 (118). In hypertonic solutions of non-electrolytes the density of elementary bodies increases appreciably.

*Response to Osmotic Influences* The changes observed in the sedimentation rates of elementary bodies suspended in solutions of different osmotic pressures vary not only with the concentration of the solute but also with the nature of the solute and the time of exposure (98). These changes are more pronounced and occur more rapidly when concentrated solutions are employed. Hypertonic solutions of sucrose remove water from the elementary bodies, this is shown by the progressive increase in sedimentation rate for an appreciable time after exposure until a maximum is reached after which the rate remains constant. In contrast, hypertonic solutions of urea first remove water from the virus particles

(the sedimentation rate increases), then urea and water diffuse into the bodies resulting in a return to their original size (the sedimentation rate drops to normal for the medium). A complete reversal of the course of events is observed when elementary bodies which have been treated with sucrose or urea are resuspended in dilute buffer solution, the sedimentation rate finally returns to the neighborhood of 4910 Svedberg units.

The behavior of elementary bodies in solutions of sucrose, glycerol and urea is similar to that of erythrocytes (98). Although analogies are tempting, one cannot conclude from this evidence alone that elementary bodies are limited by a semipermeable membrane which functions on osmotic principles. If the virus particle were a gel-like structure without a superficial membrane such as postulated by McFarlane and his co-workers (63), it might still behave in the manner observed. It would be of interest to know how structures such as tiny collodion or gelatin micelles or even large protein molecules such as edestin and the plant viruses would respond to the treatment given to elementary bodies.

*Nature of the Surface of Elementary Bodies* The surface of the elementary body is made up in part, at least, of protein material, both serological and physical data provide information on this point. Individual antibodies against three of the antigens of vaccinia, *i.e.*, L (heat-labile), S (heat-stable) and NP (nucleoprotein) are each capable of agglutinating the virus (17, 101). Therefore, both the L- and S- portions of the LS-protein molecule (94) and the reacting part of the nucleoprotein molecule are present on the surface of the virus. Electrophoretic evidence is consistent with the idea that these proteins constitute an appreciable portion of the surface of the virus. The electrophoretic mobilities of native LS-protein, of heat-denatured LS [formerly designated S, now L'S (103)] and of NP are relatively close, being 5.4, 4.4 and  $6.0 \times 10^{-5}$  cm/sec per volt/cm respectively in 0.05 M lithium veronal buffer solution, pH 7.9 (94, 101). Both elementary bodies of vaccinia and solutions of L'S move at a rate of about  $10 \times 10^{-5}$  cm/sec per volt/cm in 0.1 M lithium veronal solution pH 7.9 (93). Since electrophoretic behavior is primarily dependent on surface charge one may justly conclude that the elementary body is covered with a substance, or mixture of substances, having an electrical charge close to that of the protein and nucleoprotein antigens. This similarity in electrophoretic rate becomes more impressive when one considers that elementary bodies coated with rabbit albumin move at a much slower speed than normal whereas elementary bodies treated with a detergent that denatures the antigens and inactivates the virus move at a much faster rate (93).

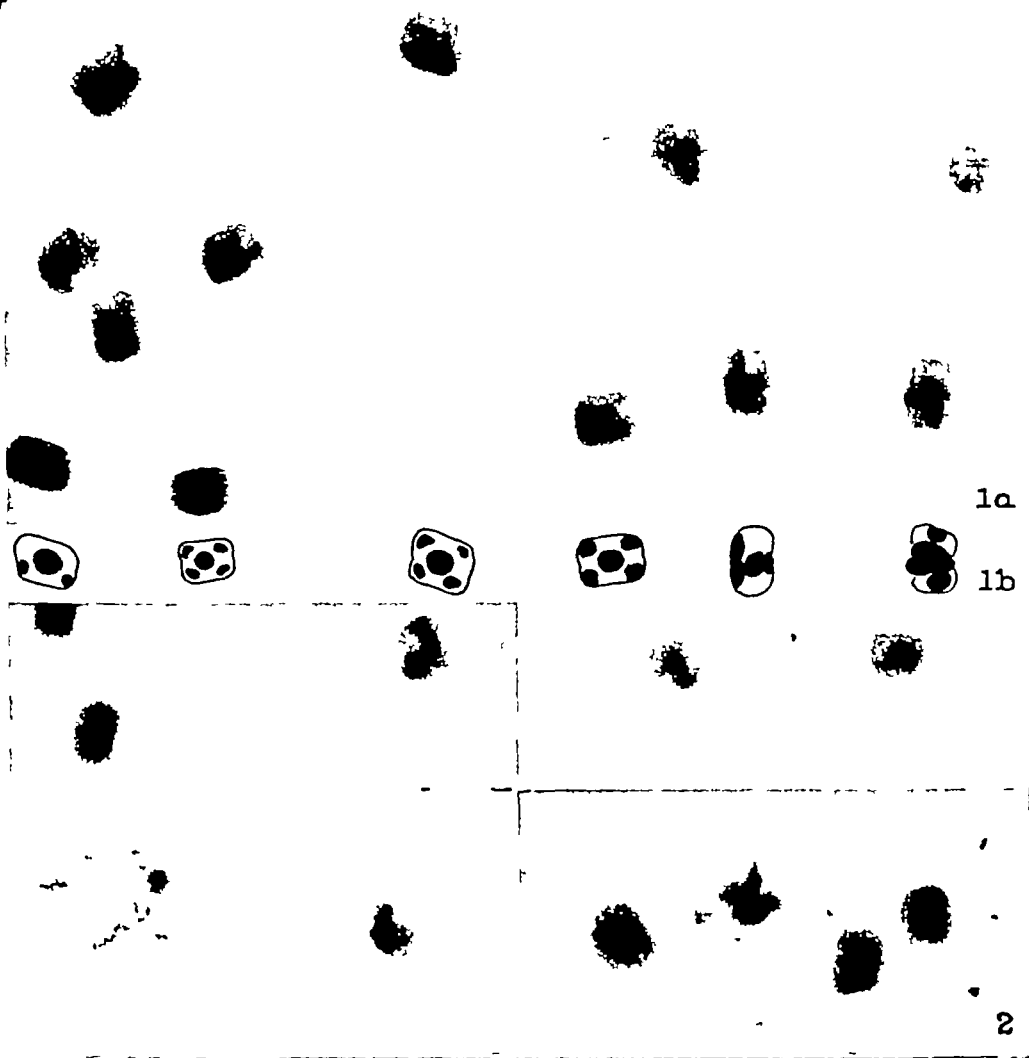
The rôle of lipids in the surface structure of the virus cannot be adequately assessed at this time. The electrophoretic mobility of elementary bodies, like their infectivity, is unaffected by extraction with ether (62, 93), however, removes only cholesterol from the bodies and does not reduce the neutral fat or phospholipid (36). Neutral fat, when extracted *in situ* by procedures which destroy its infectivity, is soluble in ether and by lipase, therefore, the failure of these procedures to diminish intact elementary bodies indicates that fat is probably

in the virus (36) Whether neutral fat and phospholipid are involved in the limiting membrane which encases the particle and which may be responsible for its response to osmotic influences remains to be determined

*Morphological Structure of Elementary Bodies* Recent electron micrographs of elementary bodies of vaccinia (30) have added much to our understanding of the structure of the virus Examined under the proper conditions the bodies show six rectangular plane surfaces so arranged that the three-dimensional shape is more brick-like than cuboidal The particles possess five areas of condensation which appear darker than the surrounding substance in the electron micrographs The centrally placed area of condensation is larger than the four areas of approximately equal size situated around it An electron micrograph of elementary bodies vaguely suggests a group of five-spotted dice Particles with a similar external shape and internal structure are not encountered in control materials Furthermore, the areas of condensation apparently represent natural phenomena since they are found under various conditions of drying, including dehydration from the frozen state It should be pointed out that elementary bodies readily adsorb protein substances on their surfaces (42, 93) and if these are not removed immediately before films of virus are prepared for electron micrography, different results are obtained Thus, inadequately washed virus particles appear oval in outline and are of homogeneous density (30) like those first observed by this micrographic technique (123) Specific antibody, unlike other adsorbed serum proteins, is tightly held even after careful washing Therefore, such treated particles are also oval and without visible areas of condensation in the micrographs (30)

Swelling and rupture of elementary bodies treated with alkali are readily demonstrated by electron micrography (30) After short exposure to dilute sodium hydroxide the bodies are larger than normal and more nearly round in outline Moreover, the areas of condensation stand out prominently since the surrounding substance is less dense than usual Treatment with stronger alkali, such as is used to extract the nucleoprotein antigen from elementary bodies (101), completely lyses the bodies Electron micrographs now show particles with irregular fuzzy outlines that frequently have wedge-shaped gaps which point in toward their centers The background substance and the areas of condensation are much less dense than normal in the lysed particles and in some pictures the internal substance has been caught in the act of streaming from the breaks in the surfaces of the bodies Such observations as these clearly indicate that elementary bodies of vaccinia have a characteristic morphological structure, that they are capable of swelling, and that their internal substance may leak out from breaks in their surfaces Figures 1 to 3 (Plate 1) represent reproductions of electron micrographs of elementary bodies of vaccinia The structure of active virus particles is illustrated in figure 1, while figures 2 and 3 demonstrate the changes produced in the bodies by alkaline treatment

The morphological structure of vaccinia is similar in many respects to that of bacteria (67, 123) and different from that of plant viruses (2) The nature of the areas of condensation found in many bacteria is not known but they may



FIGS 1-3

contain chromatin (55) In the realm of pure speculation it is amusing to connect the areas of condensation in elementary bodies with the observation of Lea and Salaman (51) on inactivation of the virus by radiation These workers performed experiments of the type employed by geneticists and concluded that the virus possessed genes Furthermore they say, "The genes are not present in a compact mass, but are either dispersed throughout the particle, or are confined to a nucleus of a diameter at least half that of the particle" The areas of condensation seen in the electron micrographs make up an appreciable part of the elementary body

*Summary of Physical Properties of Elementary Bodies* The virus of vaccinia is one of the largest members of the group of viruses, indeed, the elementary body approaches the size of rickettsia and the smallest bacteria (for a scale of comparative diameters see (113)) Elementary bodies have a brick-like shape with several areas of internal condensation Hence, their morphological structure resembles that of bacteria more closely than plant viruses The density of vaccine virus is somewhat greater than that of bacteria but considerably less than that of proteins Elementary bodies have a limiting surface structure which contains protein materials and perhaps lipoprotein as well The virus particles respond to osmotic influences in a manner analogous to erythrocytes

#### THE CHEMICAL NATURE OF ELEMENTARY BODIES OF VACCINIA

Notwithstanding an imposing array of facts concerning the chemical and physical nature of viruses which has been made available in recent years, members of this important group of disease-producing entities continue to be recognized almost wholly by their biological and immunological activity In the forty years prior to 1935, during which time the filterable nature of viruses was known, these agents have been variously considered as invisible, involutinal forms of ordinary bacteria and protozoa, as toxins, enzymes, or end-products of cell metabolism, and as new and uncharacterized types of invisible living agents

In 1935, Stanley (111) and shortly thereafter, Bawden and coworkers (3) isolated a macromolecular, para-crystalline nucleoprotein from diseased tobacco plants which possessed all the properties of the disease-producing agent This report was followed quickly by others on the isolation of macromolecular nucleoproteins associated with the disease-producing agents of aucuba mosaic (113), rabbit papilloma (5), bushy stunt of tomato (3), chicken tumor (9), and staphylococcus bacteriophage (69) The experiments of Claude (10), however, in isolating from normal chick embryo a macromolecular nucleoprotein chemically indistinguishable from the tumor-producing agent, and the experiments of Kalmanson and Bronfenbrenner on the non-nucleoprotein nature of bacteriophage (46), have served to temper with some caution the ready acceptance of evidence thus far produced that many of these substances represent virus in its true state In certain instances, however, the evidence that the agent has been secured in pure state is unequivocal, and the material meets the most rigid standards for purity and homogeneity

At the moment, the problems facing the worker in virus chemistry are those

which face all investigators of the structure and properties of protoplasm. To the enzyme chemist there is much in viral agents to remind him of enzymes, to the chemist concerned with hormones and toxins there is much in the inherent properties of viruses to remind him of these substances. Moreover, to the chemist working with genes and other active constituents of protoplasm, there is the almost virginal field of protoplasmic structure as related to propagation and mutation, two of the most apparent properties of viral agents.

Of the animal viruses which have been concentrated by chemical and physical means during the past few years, only one, the virus of vaccinia can lay much claim to purity. With respect to preparations of elementary bodies, criteria of purity other than those of centrifugal and electrical homogeneity have been sought. Elementary analyses, although incapable of fine differentiation, do indicate, however, that while material discarded at similar stages in the centrifugal concentration of the virus may differ markedly from lot to lot, a final product is obtained which upon analysis agrees closely with other lots of virus prepared in the same manner. When this stage of purity is reached, further washing and centrifugation do not alter materially the analysis of these elementary constituents. Other criteria of purity, such as crystallization and phase rule, procedures and techniques which were of such significance in establishing the purity of tobacco mosaic virus, are at the moment inapplicable to vaccine virus, the units of which are of the order of 250 m $\mu$  in size, and extremely insoluble in all but the most drastic reagents.

*Basic Analysis of Purified Vaccine Virus* In 1935, Hughes, Parker and Rivers (43) performed certain preliminary analyses which indicated that elementary bodies of vaccinia possessed, in the main, the constituents of ordinary protoplasm, namely, nitrogen, fat, and ash. Further studies were not done at that time, however, because the purity of the preparations was a matter of some doubt. Hoagland, Smadel and Rivers (36) have since confirmed and extended these analyses, finding a somewhat greater quantity of nitrogen and smaller quantities of lipid. Values for the basic constituents of elementary bodies of vaccinia are listed in table 1. These include analyses for nitrogen, phosphorus, lipids and carbohydrates on eleven lots of purified virus with high infective titers and high ratios between infective units and number of elementary bodies.

Analyses for basic constituents show but few important differences between elementary bodies of vaccinia and certain of the purified plant viruses. Actually, there is more variation in the elementary analyses among the individual plant viruses than exists, for instance, between the elementary bodies of vaccinia and tobacco-mosaic virus. Stanley's tobacco-mosaic virus contains carbon 50 per cent, hydrogen 7 per cent, nitrogen 16 per cent, sulfur 0.24 per cent and phosphorus 0.6 per cent (114), values which are a near approximation to analytical data obtained for vaccine virus (36). Tobacco ring spot virus, on the other hand, contains almost 4 per cent of phosphorus. There is no lipid in crystalline tobacco virus, while preparations of elementary bodies regularly contain 5 to per cent lipid. It is when comparisons of constituents, as opposed to elemen

chemical analyses, are made that certain very important differences between elementary bodies of vaccinia and tobacco-mosaic virus emerge

*The Nature of the Lipids in Elementary Bodies* Data on the total lipid as well as on the amounts of neutral fat, phospholipid and cholesterol in preparations of elementary bodies are summarized in table 1. Cholesterol apparently occurs in the unesterified form (36) and may be simply adsorbed on the surface of the virus for it is readily removed by extraction with ethyl ether (36, 63), a procedure which does not inactivate the virus. Neutral fat and phospholipid are not decreased by such treatment, however, these lipids are extracted from elementary bodies by mixtures of alcohol and ether. This more drastic manipu-

TABLE 1  
*Analyses of elementary bodies of vaccinia*

LOT	IU EB RATIO*	PHOS PHORUS	TOTAL NITROGEN	LIPID				REDUCING SUGARS AFTER HYDROLYSIS†
				Total lipid	Cholesterol	Phospholipid	Neutral fat	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
46	1 7 6	0 59	15 3	5 1	1 1	2 1	1 9	3 0
47	1 8 8	0 59	15 6	6 1	2 2	1 8	2 1	2 3
48	1 2 7	0 50	15 1	5 0	1 1	2 0	1 9	3 2
53	1 4 7	0 58	15 6	5 0	1 0	2 2	1 8	2 0
55	1 3 0	0 49	15 3	4 3	1 0	1 7	1 6	2 6
56	1 3 7	0 59	14 8	6 1	1 2	2 5	2 4	3 5
57	1 9 2	0 58	15 2	5 3	1 1	2 2	2 0	3 0
58	Not done	0 57	15 2	4 9	1 0	1 9	2 0	3 1
66	1 4 2	0 59	14 9	8 1	2 1	2 8	3 2	2 5
69	1 3 7	0 62	15 4	6 5	1 8	2 1	2 6	2 3
70	1 1 5	0 57	15 5	6 2	2 0	2 5	1 7	3 5
Average	1 4 9	0 57	15 3	5 7	1 4	2 2	2 2	2 8

Values of all chemical analyses are expressed as per cent of dry weight

\* IU EB = ratio between infective unit and number of elementary bodies

† Expressed as glucose

Data reported by Hoagland, Smadel, and Rivers (36)

lation inactivates the virus but whether loss of infectivity occurs from withdrawal of these lipids or from some other action of alcohol cannot be stated (36, 63). Further studies in the attempt to estimate the significance of neutral fat in the preparations of virus were made by Hoagland, Smadel and Rivers (36) by means of enzymatic digestion. Partially purified pancreatic lipase failed to hydrolyze the neutral fat in preparations of active elementary bodies, but this same enzyme readily digested the fat after it had been extracted from the virus by alcohol and ether. This observation indicates that the neutral fat present in preparations of active virus is bound in some manner so that it is incapable of being hydrolyzed by lipase. Furthermore, it strongly suggests that the neutral fat is an essential constituent of the elementary body in contrast to cholesterol which may be an extraneous substance carried over from the host

tissue. Conclusive evidence regarding the importance of phospholipid in the economy of the virus is not at hand but the available data suggest that phospholipid, like neutral fat, is a constituent of the elementary body (36).

Considerable interest has been aroused by the lipid of the elementary body because if lipid is an integral part of this virus, then the molecular concept of viruses cannot be applied to the agent causing vaccinia. Of course the virus might still be assumed to be a complex lipoprotein macromolecule but such an assumption would rob the term "molecule" of much of its inherent meaning.

*The Nucleic Acid of Elementary Bodies* Nucleic acid has been one of the most important constituents of viruses thus far studied. With the possible exception of the coliphage isolated by Kalmanson and Bronfenbrenner (46) no viral agents have been obtained which are free of nucleic acid. Even in the purified coliphage, the minute trace of phosphorus present may conceivably have come from nucleic acid, although these workers consider it a very remote possibility. Otherwise, viral agents may contain as much as 40 per cent of this "common denominator" substance.

Smadel, Lavin and Dubos have shown that with dilute sodium hydroxide there could be extracted from elementary bodies of vaccinia a material giving a positive Feulgen reaction, indicating the presence of thymus nucleic acid (97). Following this preliminary report, Hoagland, Lavin, Smadel and Rivers were able to isolate and characterize this substance as thymus nucleic acid by an analysis of its component parts (37). From 500 mg of elementary bodies approximately 15 mg of pure nucleic acid were isolated. This substance gave an elementary analysis comparable to that calculated for a tetra-nucleotide. It gave a strongly positive Feulgen reaction and an intense blue color with diphenylamine. Crystalline adenine and guanine were recovered from the material, and a qualitative test for thymine with *o*-amino-benzaldehyde was positive. The ultraviolet absorption spectrum was characteristic of nucleic acid.

Further evidence that the nucleic acid of elementary bodies is of the thymus type is evident from studies on its resistance to ribonuclease (37). Crystalline ribonuclease, shown to be active in the depolymerization of yeast nucleic acid, was inactive when incubated with nucleic acid isolated from elementary bodies of vaccinia. The slight amount of depolymerization which seemed to occur regularly may have been referable only to some spontaneous change in the nucleic acid in the presence of buffer, or to the presence of traces of ribonucleic acid in the preparation. The negative Bial's test on the material, however, indicated that no more than a trace of ribonucleic acid was present, if any at all. It must be remembered, however, that ribonucleic acid is more soluble than the thymus variety, and small amounts, if originally present, may have been removed by the repeated washings employed in the preparation of the virus.

The stable blue color produced by diphenylamine with thymonucleic acid in the presence of glacial acetic and sulfuric acids, can be obtained with elementary bodies of vaccinia without preliminary isolation of the nucleic acid, if crystalline pepsin is used to digest away the protein portion of the elementary body. Comparison of the color produced by elementary bodies and diphenylamine reagent

with that produced by c p thymonucleic acid shows that the elementary body contains an average of 5.6 per cent nucleic acid. This compares favorably with the value for nucleic acid calculated from the non-lipid organically bound phosphorus in the whole elementary body.

In tobacco-mosaic virus, the nucleic acid seems to exist as a conjugated nucleoprotein and together with the protein accounts for the entire virus molecule. In the elementary body, the picture is complicated by the presence of a number of other constituents to be described later which in character and concentration are very similar to those of ordinary protoplasm.

Loring (56) has shown by chemical isolation of guanine, cytosine, adenine, and uridylic acid that the nucleic acid from tobacco-mosaic virus belongs to the group of ribo- or yeast-nucleic acids, although it differs in certain respects from all known nucleic acids. The evidence that the nucleic acid from vaccine virus belongs to the desoxyribose or thymo-nucleic acid group is equally good. Whether this is an important difference between animal and plant viruses cannot be stated since the work of Claude has shown that the nucleic acid from his chicken tumor agent is of the yeast variety (9). Recent work by Claude (11) however, shows that in his material thymo-nucleic acid may also be present in small amounts. It may be added that studies on the nucleic acid of the chicken tumor virus can by no means be regarded as final until a higher degree of purification of this material has been achieved.

With respect to the protein component of vaccine virus, particularly with reference to the amino acid constituents, no important work has yet been done. The minute amounts of virus obtainable make work of this nature exceedingly costly and forbiddingly difficult. Knight and Stanley (48), however, have been able to do some very critical work on the isolation and study of the amino acid components of tobacco-mosaic virus. Amino acids isolated thus far from this virus include arginine, aspartic acid, cysteine, glutamic acid, leucine, lysine, phenylalanine, proline, serine, tyrosine, and tryptophane. Alanine, histidine, and glycine appear to be absent or to occur in amounts too small to be detected. Future work may reveal certain important differences between the amino acid constituents of different strains of the virus. Preliminary results obtained by Knight and Stanley have already indicated that specific differences in the amino acid content of closely related strains of the plant viruses do occur (48).

*The Effect of Proteolytic Enzymes on Elementary Bodies* One property of vaccine virus which makes work on its constituents exceedingly difficult is its extreme insolubility in all but the most drastic reagents. Extensive studies on the physical properties of the elementary body of vaccinia, its resistance to autolysis and its maintenance of intact structure, even after months of storage in the cold, have revealed a stability not shared by most other animal viruses. Merrill (64) has indicated a remarkable resistance of the elementary body to pure proteolytic enzymes. An explanation of the lethal effects of proteolytic enzymes for elementary bodies of vaccinia reported by earlier workers is suggested by the studies of Antonette Pirie (80) who showed that traces of fatty acids contained in commercial pancreatin preparations are exceedingly virucidal,

these fatty acids may have accounted for the effects which had been previously attributed to the enzymes themselves

Hoagland, Ward, Smadel and Rivers (38) in order to learn something of the more labile constituents of the purified virus, turned to a survey of the effects of various proteolytic enzymes in the hope that some means might be found for putting the virus into solution without destruction of the heat- and alkali-labile components. In the hands of these workers, no immediate effects of crystalline trypsin, chymotrypsin, or carboxypeptidase on the elementary body of vaccinia could be demonstrated at physiological pH and temperature. Purified cathepsin was likewise ineffective. Pepsin, at a pH which in itself inactivated the virus, resulted in a rapid release of free amino nitrogen.

In view of the close association between vaccine virus and host cells, it would seem that plant enzymes might more logically be expected to effect proteolysis. With this in mind a survey of the effects of plant proteolytic enzymes on vaccine virus was made, with amino nitrogen determinations performed as a check on hydrolysis and with simultaneous studies on infectivity and staining reaction. Papain, in contrast to the animal proteolytic enzymes, after a short time effected startling changes in the elementary body, and within two hours the infectivity of the virus had totally disappeared. Papain, activated with cyanide, produced almost simultaneously four important changes in the elementary body: 1. Structural disintegration, as revealed by Morosow's stain, 2. Destruction of infectivity as revealed by animal inoculation of the partially hydrolyzed material, 3. Loss of antigenicity as revealed by the failure of the partially hydrolyzed material to be precipitated with hyperimmune sera produced by inoculation of intact elementary bodies, 4. Release of free amino groups measurable with nitrous acid. That the lethal effect of papain is on the native virus proper and not an indirect effect on the cells of the host is shown by the infectivity of virus-papain mixtures before and up to two hours after incubation with the enzyme (38).

No conclusions with respect to the "living" or "non-living" nature of the elementary body can be drawn from the experiments with proteolytic enzymes since both viable and non-viable protoplasm may be attacked by plant proteolytic enzymes. Thus, certain annelids undergo decomposition in ficin solutions (87), and recently digestion of the living cornea has been reported to occur from ficin applied to the eyes of rabbits (65). Crystalline ficin has no demonstrable effect on elementary bodies of vaccinia (38). Whether crude ficin, which is believed to be a mixture of proteolytic enzymes, would have been effective was not determined.

*Biological Catalysts in Vaccine Virus* The foregoing studies, although incomplete in certain respects, have indicated a complexity in the structure of vaccine virus which is not shared by simpler viruses of plant origin. While interesting, it is doubtful whether continuation of this mode of attack would yield sufficient information at this stage of our knowledge of animal viruses to justify the expenditure of time and of the relatively enormous quantities of costly virus needed for a strictly organic chemical investigation. Accordingly

we may turn to studies designed more along the line of a physiological approach, attempting to examine some of the notions which have been advanced concerning virus metabolism. Does the virus depend partially or wholly for its substrate on the animal host cell, or does it possess fragments of rudimentary metabolic systems?

One of the most attractive of the notions having to do with the metabolism of vaccine virus is the hypothesis that, as an obligate parasite, it may possess only a portion of the enzyme and respiratory systems necessary for its survival, relying in the main upon its intracellular environment for complementary systems which enable it to multiply within the host cell (40). Certainly the size and the chemical and antigenic complexity of the elementary body, which make it resemble in some respects minute bacteria, make this hypothesis tenable.

Parker and Smythe (76) performed certain experiments which were interpreted as showing that the elementary body did not possess an independent metabolism, inasmuch as no oxygen utilization or acid production could be demonstrated by the virus in the presence of hexosephosphate and "respiratory factor" from red blood cells. Much has been learned about respiratory systems since then, and it is clear that these initial studies must be greatly extended before negative conclusions concerning the respiration of elementary bodies can be safely drawn.

Recent observations of Warburg, Krebs, Keilin and others (29) have demonstrated that while any one, or more than one, of a multiplicity of dehydrogenases may participate in a cellular respiratory reaction-chain, certain well-defined organic catalysts within the cell take up key positions in the system of energy exchange which begins with the oxidation of substrate and ends in the reduction of molecular oxygen. Of these catalytic systems, flavin-adenine-dinucleotide, the diphosphopyridine and triphosphopyridine nucleotides, and cytochrome play important and fairly well-understood roles. In other words, these catalysts may be said to act as channels in a cellular oxidation-reduction chain for the transfer of hydrogen from an oxidizable substrate to molecular oxygen, with the formation of water or peroxides.

If the hypothesis that the elementary body may contain a partial respiration system is warranted, it is obviously simpler experimentally to look for members of these well-defined catalytic substances than to search for dehydrogenase function by means of specific substrate reactions. Likewise, these systems represent stages in the enzymatic degradation of substrates where large quantities of free energy may be released and rendered available for virus synthesis.

With these ideas in mind, Hoagland, Ward, Smadel and Rivers (40) undertook a systematic search for certain of these catalytic substances in purified elementary bodies of vaccinia, beginning with cytochrome *c*. Respiration in aerobic organisms is known to go in part, if not chiefly, through the cytochrome system which acts as a reversible oxidation-reduction link between oxygen and certain enzyme systems which have become reduced in the process of oxidation of cell substrates. So far as it is now known, all cells that are able to use molecular oxygen contain one or more members of a group of protein porphyrins with iron in tetra-pyrrolic combination. These substances, known collectively as the cy-

tochromes, have been shown to participate in reversible oxidation-reduction processes within the cell. Spectroscopic observations made on numerous specimens of virus failed to reveal, in the presence of suitable reducing agents, the bands of cytochrome in the visible or ultra-violet region of the spectrum. Although spectroscopic methods are extremely sensitive in the detection of reduced cytochrome, as shown in Keilin's classical studies, it was entirely possible that the opalescence of the virus suspension had masked faint bands of cytochrome. In order to check this point a potent cytochrome oxidase was prepared from beef heart, according to the method of Keilin, and set up with vaccine virus in the presence of several suitable hydrogen donors, such as hydroquinone and *p*-phenylenediamine. No appreciable oxygen uptake over controls was noted in a system of virus, cytochrome oxidase and *p*-phenylenediamine, although the enzyme was extremely active in the oxidation of pure cytochrome *c* preparations in the presence of the enzyme and the same hydrogen donors.

During attempts to demonstrate cytochrome activity of the elementary bodies these authors noted that alkaline solutions of vaccine virus gave an intense fluorescence in the presence of ultraviolet light, and furthermore, that this fluorescence could be demonstrated with proper filters to be maximum around 3500 Å. Increased alkalization and heat caused this fluorescence to disappear. Likewise, solutions standing in strong light tended to lose their fluorescence and regain it upon standing in the dark. It was apparent that this peculiar behavior could be accounted for if the fluorescent material were a flavin compound—a substance which is just below cytochrome in the series of known cellular catalysts arranged in order of their descending oxidation-reduction potentials.

A fraction which carried the major portion of the fluorescent substance of the virus was dissociated from the virus by treatment with heat and 50 per cent alcohol at pH 4. Preliminary studies on this fluorescent material showed that it had certain physical and chemical properties in common with riboflavin, however, the substance could not be obtained in sufficient quantities or in a sufficiently pure state for identification by direct means. Recourse to two indirect methods of identification enabled Hoagland, Ward, Smadel and Rivers (41) to establish the flavin nature of this fluorescent material. One of these methods was Krebs' technique for the estimation of flavin-adenine-dinucleotide by means of the protein of d-amino acid oxidase (49), and the other was the microbiological assay for riboflavin of Snell and Strong (106) which employs *Lactobacillus casei* *E*. By means of the latter technique preparations of purified virus were shown to contain between 1.1 and 1.5 mg of riboflavin per 100 g of material. This amount is comparable to that present in muscle and in some bacteria but is distinctly lower than that in most yeasts (41).

All attempts to remove the flavin by means which did not inactivate the failed, namely, by repeated washing with buffers ranging from pH 6 filtration by means of the Coolidge apparatus, or electro dialysis. Flavin could be removed by means of heat and alcohol at pH are known to dissociate flavin-adenine-dinucleotide in solution (41).

The demonstration that preparations of elementary bodies of vaccinia contain constant amounts of flavin-adenine-dinucleotide is interesting, but proof is still lacking that this substance is an integral part of the elementary body and that it functions in the metabolism of the virus. If one assumes for the moment that flavin is a constituent of the virus then the flavin might be thought of as part of the catalytic system of the virus which enables it to divert energy for its own needs from the oxidation-reduction chains of the host cell. No experimental evidence can be offered for such an hypothesis but it may be useful as an idea on which to base the search for additional components of this enzyme system and of other systems in the virus in the hope of gaining a fuller understanding of metabolism of the virus.

*The Presence of Biotin in Elementary Bodies of Vaccinia* Biotin is among the important factors which play a major role in bacterial metabolism, particularly in the metabolism of anaerobes. This substance has taken on renewed interest lately because of its identification with coenzyme R (1), a factor required by many bacteria, and with vitamin H (21), an essential substance for mammals which is neutralized by raw egg-white. It is perhaps the most active catalytic substance known and can be detected as a growth stimulant to yeast and certain bacteria in amounts of the order of  $10^{-6}$  micrograms.

By a technique similar to that described by McDaniel, Woolley and Peterson (61), Hoagland, Ward, Smadel and Rivers (39) have shown that elementary bodies of vaccinia contain relatively large quantities of a substance capable of stimulating the growth of *Clostridium butylicum* in a medium devoid only of biotin. Moreover, partial hydrolysis of the virus by 4N sulfuric acid or by normal alkali gives rise to increased quantities of the substance, which on the basis of studies similar to those made on liver, egg yolk, etc. is believed to be biotin (105). Although elementary bodies alone, with no other treatment except autoclaving in a synthetic medium prior to inoculation with organisms, yield fair amounts of the growth factor for *Clostridium butylicum*, partial hydrolysis by acid or alkali renders a marked increase of the amount available. This observed increase in biotin content is probably analogous to the greater yield of biotin noted by Gyorgy in vitamin H following acid hydrolysis.

*The Metal Component of Elementary Bodies of Vaccinia* The discovery that purified elementary bodies of vaccinia contain relatively large amounts of copper came about through a search for a possible cytochrome component (40). In the process of trying a variety of substances as hydrogen donors, it was observed that a relatively enormous oxygen uptake could be demonstrated in the Warburg vessel when vaccine virus was mixed with cysteine. The reaction was completely blocked by 0.005 molar potassium cyanide, but was unchecked by  $\alpha$ ,  $\alpha'$ -dipyridyl, a fact which rules out the presence of iron catalysts. The effectiveness of certain inhibitors, such as diethyl-dithiocarbamate, indicated that the catalytic substance was copper. This was confirmed by emission spectra obtained from the dry virus. Scrupulous care, exercised in the preparation of virus and in the selection of copper-free reagents, produced no diminution in the copper content of 0.05 per cent. Moreover, electrodialysis over a pH range from 6 to 10 for 24

hours failed to remove any part of this element. Since in no instance could steps in the method of purification of the virus be shown to be responsible for the introduction of the copper, and since it was not possible to remove it by electro-dialysis, ultrafiltration, or repeated washing, it may be regarded at the moment as a component either bound or closely associated with the virus particle.

The emission spectrum of vaccine virus, aside from furnishing unequivocal proof of the presence of copper, has been helpful in establishing the purity of elementary body preparations (40). Crude virus can be shown by emission spectra to contain a number of metallic compounds. These include iron, manganese, magnesium and zinc in considerable amounts. As purification proceeds, however, all detectable traces of metals other than copper disappear. In the final virus product no traces of other metals can be demonstrated on as much as 20 milligrams of dry virus. Copper, however, is concentrated at least 25-fold.

The experimental evidence clearly warrants the assumption that copper is an integral part of the virus and that it is closely bound with some constituent of the elementary body. The function of copper in the virus has not been determined but it is tempting to speculate on the possibility that this element is bound to a protein component of the virus and serves as an oxidation catalyst in the respiratory activity of the elementary body, polyphenol oxidase and tyrosinase are known examples of copper-protein combinations with enzymatic activity.

*The Nature of the Enzymes Associated with Elementary Bodies of Vaccinia*  
Macfarlane and Salaman (60) have reported the presence of phosphatase, and catalase in elementary bodies of vaccinia, but they were unable to demonstrate dehydrogenase activity, although the reaction with a variety of substrates was studied. They found vaccine virus to lack zymohexase, enolase, gluconidase, and nucleosidase. Both phosphomonoesterase and phospho-diesterase were present, as well as ribonuclease. Fresh virus preparations were also found to possess an enzyme which produced a rapid hydrolysis of muscle adenylic acid (59). The assumption by these authors that the enzyme activity of their virus preparations was due to the virus and not to tissue contaminants was based on the observation that certain of these enzymes were concentrated during the process of purification, while others, such as the dehydrogenases, which were present in high titer in the tissues and cell fragments from which the virus was separated, were absent from the final virus product. These authors considered the possibility that the presence of these enzymes may have been due to surface adsorption, but dismissed the idea because frequent washings failed to elute them.

Hoagland, Ward, Smadel and Rivers (42) have repeated certain of the studies of Macfarlane and Salaman, and have extended them to other enzymes which are found in the host tissues from which the virus is prepared, and which might, therefore, be associated with the purified virus. At the same time, tests were made of the adsorptive capacity of elementary bodies for these enzymes. Elementary bodies on which the titer of phosphatase had been determined, were suspended in a dilute solution of purified phosphatase for 1 hour at 20°C. Virus was recovered by centrifugation and washed until the superna

waters no longer gave a test for phosphatase, at which time the virus was retested for phosphatase activity. A three-fold increase in activity was exhibited by the phosphatase-treated virus. Similar experiments were performed with catalase and lipase. The increase in catalase activity was too great to be measured accurately, and the lipase titer of the virus was found to have increased 10- to 15-fold. Although it has not been possible to design experiments which prove that the enzymes found in the purified virus are adsorbed from the tissue and cell fragments from which the virus is prepared, the fact that the virus will take up large quantities of certain enzymes from dilute solution makes a strong case for the adsorption hypothesis. Dehydrogenases, which occur in cells generally, and which might therefore be expected to appear as contaminants, seem to be absent from the purified virus. If present initially, the lability of these enzymes might well preclude their surviving the several steps employed in the purification of the virus. Until methods can be devised which are capable of distinguishing between enzymes which represent an integral part of the virus and those which exist as host contaminants, it would seem that the problem of enzymes in purified animal viruses is impossible of solution.

*Summary of Chemical Constituents of Elementary Bodies and of the Enzymes Associated with Them.* A study of the chemical constituents shows that elementary bodies of vaccinia, unlike the crystalline plant viruses, are complex entities, and that they contain many of the constituents of ordinary protoplasm, and in concentrations which do not differ materially from those found in the host tissues. Among the substances which seem to be integral, or at least closely associated components of the elementary body are nucleoprotein, fat, carbohydrate, copper, flavin and biotin. The presence of these last three constituents would indicate that some rudimentary system of respiration for this particular virus may exist although no substrate capable of sustained activation by these catalytic substances has been found. It must be borne in mind, moreover, that as yet no enzyme protein (such as phosphatase, catalase or lipase) has been found in purified elementary bodies which is not at the same time a known constituent of host tissue, and the presence of which might not be accounted for on the basis of its having been adsorbed from the host cells.

#### ANTIGENS OF VACCINIA

Many of the important immunological facts in the variola-vaccine problem were established long before the nature of those infectious agents was understood. For example, our main interest in the subject of vaccinia stems from Jenner's report (44) in 1798 of the protection of human beings against variola by a previous infection with the virus of cowpox. In addition, Raynaud (1877) demonstrated that immunity to vaccinia is passively transferred by convalescent serum (84) and Sternberg (1892) showed that such serum is capable of inactivating the virus *in vitro* (117). Furthermore, Tanaka (1902) and Jobling (1906) found that antiserum flocculates preparations of virus (119) and fixes complement in its presence (45). Gordon (27) did much to bring order out of the confusion that existed prior to 1925 regarding the flocculation and complement-fixation reac-

tions in this disease. Recent observations made on highly purified materials have illuminated many aspects of the problem of the antigens of vaccine, however, the most important phase, *i e.*, that dealing with the antigen or antigens responsible for the development of immunity and neutralizing antibody, has been advanced relatively little during the past decade.

The virus of vaccinia contains a number of antigenic substances which elicit the production of specific antibodies in hyperimmune animals. Gordon's early observation (27) on immune serum which dealt with the lack of correlation between neutralizing substances and antibodies capable of flocculating or fixing complement with his crude preparation of virus suggested the presence of more than one antigen. Craigie and Wishart established the existence of a heat-labile (L) and heat-stable (S) soluble substance which occur in combination (LS) and are found on the surface of the elementary body as well as in solution free from the virus (17). These same workers also showed that another antigen, the agglutinin X, makes up part of the surface mosaic of the virus (19). More recently, Smadel, Rivers and Hoagland (101) have extracted a nucleoprotein antigen (NP) from the virus. A discussion of the antigens of vaccinia will be simplified if it is emphasized at once that LS (73) and NP (101) are not capable of inducing the production of appreciable resistance or of significant amounts of neutralizing antibody in normal rabbits injected with them. On the other hand, antibody against L, S, NP or X is capable of agglutinating the virus (14, 19, 101). A brief discussion of the more important properties of each of the antigens of vaccinia follows.

*LS-Antigen* The intimate association of the heat-labile and heat-stable soluble substances of vaccinia was recognized by Craigie and Wishart (18). These workers demonstrated that L- and S-antibodies are separate antibodies but that the absorption of preparations rich in L- and S-antigen with either antibody removes both antigenic substances. Their interpretation of these observations, namely, that L and S are different antigenic components of a complex LS-antigen, has been substantiated by Shedlovsky and Smadel (94) who found that a single protein molecule possesses all of the serological properties of L- and S-antigen. LS-antigen is specific for variola-vaccinia virus. It is found in material prepared from human variolar crusts (16), and from egg membranes infected with the virus of variola (50), furthermore, it occurs in vaccinal infected tissue obtained from the calf, rabbit, guinea pig (15) and egg membrane (104). LS-antigen derived from the various sources is indistinguishable by serological methods. The presence of this substance in other closely related pox diseases *e g.*, alastrim, horse pox, etc., remains to be determined. Craigie's opinion that the soluble antigen of vaccinia is directly elaborated by the virus and thus analogous to bacterial antigens (13) has seemed reasonable to us (104), but it has been questioned by others (90). LS-antigen and its antibodies are the substances principally involved in serological techniques employed for diagnosis of human vaccinia and variola (16).

The isolation of pure LS-protein (94) has made it possible to establish the serological, chemical and physical properties of this substance with considerable ac-

curacy The instability of the L-portion of the antigen delayed the acquisition of knowledge concerning its nature Nevertheless, Craigie and Wishart (18) developed a satisfactory method for concentrating LS-antigen, and Parker and Rivers (75) were able to obtain S-antigen [now designated L'S] in a highly purified state and to demonstrate its protein nature Shedlovsky and Smadel (94) separated a protein from filtrate of infected rabbit skin which possessed all the serological activity of the starting material and which was homogeneous on electrophoresis and also on ultracentrifugation With their co-workers (103) they have characterized the LS-protein as follows density = 1.39, specific volume = 0.72, diffusion constant =  $1.5 \times 10^{-7}$ , sedimentation constant = 4.3s (Svedberg units), all at 4°C, the electrophoretically determined isoelectric point is pH 4.8 Chemical analyses show 15.7 per cent nitrogen but no lipid, nucleic acid, phosphorus or glucosamine The LS-antigen appears to be an elongated protein molecule with a molecular weight of 240,000 and an axis ratio of approximately 30:1 (103)

### Serological activities of LS-antigen and some of its degradation products




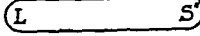
Native	Heated	Heated with alkali	Digested with chymotrypsin
			
Precipitates with both L- and S-antibodies	No precipitation with L-antibody Inhibits L-antibody Precipitates with S-antibody	No reaction with L-antibody No precipitation with S-antibody Inhibits S-antibody	Precipitates with L-antibody No reaction with S-antibody

Fig 4

LS-protein provides an example of a single molecule that is capable of eliciting two distinct antibodies It is of interest in another respect, namely, two levels of degradation are demonstrable for both the L and S parts of the molecule The property of precipitating with the corresponding antibody is lost in the first stage of serological degradation but the power of inhibiting this antibody remains, in the next stage, serological activity disappears (100) A series of degradation products that have various combinations of native and degraded parts can be prepared from pure LS by treatment with heat, alkali (94) and enzymes (96) Data on several of the more important of these are summarized in figure 4 It will be noted that LS'' precipitates only with L-antibody and L'S only with S' antibody The demonstration of degradation products of LS-protein which, on the basis of precipitin tests alone, appear to represent individual L and S substances, has clarified earlier confusion regarding the relationship of these two antigenic substances

*NP-Antigen* The nucleoprotein antigen of vaccinia (101) constitutes an appreciable part of the mass of the elementary body On the basis of weight about

half the substance in virus particles is dissolved by dilute alkali (63, 97), and at least 90 per cent of the extracted material consists of NP-antigen (101). The nucleoprotein is soluble at pH values above 8.0 and is partially or completely insoluble in the pH range between 4.5 and 7.5. Therefore, serological studies with this antigen are most conveniently carried out in solutions buffered at pH 8.0 to 8.6.

Precipitins that react with NP appear in the sera of members of several species of animals following hyperimmunization with active virus. Furthermore, rabbits repeatedly injected with heat-inactivated elementary bodies or with non-infectious alkaline extracts of virus develop precipitins for NP. Absorption experiments indicate that NP-antibodies differ from L- and S-antibodies and from the residual agglutinin, X. Moreover, the neutralizing power of hyperimmune serum is not reduced by the absorption of NP-antibodies. Finally, normal rabbits following repeated injections of NP-antigen fail to develop either immunity to infection or appreciable amounts of neutralizing antibody. These immunological data demonstrate that NP is a distinct antigen of vaccinia and that, in its present form at least, it is not the substance responsible for immunity and neutralizing antibody (101). In early experiments the heat stability of NP-antigen together with its precipitation by S-antisera, which were then thought to contain only S-antibody but which were subsequently shown to possess NP-antibody in addition, misled us into thinking that the alkaline extracts of virus contained considerable amounts of S-antigen and that this S-protein occurred in combination with thymus nucleic acid (97).

The nucleoprotein antigen is relatively stable since it retains its serological activity and immunizing properties for months when stored in the cold. Moreover, heating at 56°C for one-half hour causes no appreciable change in the appearance or in the precipitin or complement-fixation titers of solutions of the antigen, provided the pH is kept near 8.6. Similarly, little if any activity is lost when it is repeatedly precipitated and redissolved by varying the pH of the medium. Solutions containing one part in 400,000 of the nucleoprotein give a precipitin reaction with optimal amounts of NP-antibody (101).

Chemical analyses show that NP-antigen contains 14.5 per cent N, 1.8 per cent P, and 6 per cent nucleic acid of the thymus type. The electrophoretic mobility of NP is slightly different from that of LS or L'S examined under similar conditions (101).

*X-Agglutinin* Relatively little is known about this substance except that it is present in active virus and elicits the production of an antibody in certain hyperimmune rabbits. Sera from such animals still agglutinate elementary bodies after all demonstrable L- and S-precipitins have been removed. In contrast, sera from other hyperimmune rabbits are exhausted of agglutinins by absorption with LS-antigen. Craigie and Wishart (18, 19) designated the residual agglutinating antibody in the first type of serum as X-agglutinin. Smadel, Rivers and Hoagland (101) pointed out that NP-antibody might be considered as X-agglutinin since it is not removed from antisera by treatment with LS-antigen. However, they found that hyperimmune sera freed of L-, S- and NP-

antibodies still possess some agglutinating power, therefore, they redefined the X-agglutinin as the aggregating substance or substances left in antiserum after the removal of the three precipitins

The reaction attributed to X-agglutinin might result from the incomplete absorption from antiserum of the precipitating antibodies since small quantities of these antibodies might be detectable by the delicate agglutination technique but not by the precipitin reaction. This possibility has been ruled out in part by the observation (101) that complete absorption of S-precipitins removes all agglutinins for collodion particles coated with L/S-antigen. In the future, studies on X-agglutinin should be carried out with sera that are shown to be free of antibodies which aggregate inert particles coated with LS and with NP. Since antisera absorbed free of L-, S- and NP-antibody retain X-agglutinins and neutralizing substances (101), one wonders whether these last two substances are related or identical. It can only be said that Salaman (91) and Downie (20) have reported that the absorption of hyperimmune sera with adequate quantities of elementary bodies removes both agglutinins and neutralizing substances. Their experiments are surprising, however, in that the absorbed sera contained antibodies capable of reacting with dermal filtrate (crude LS-antigen). One would think that the presence of even small amounts of L- and S-antibodies in the sera would be sufficient to aggregate elementary bodies. It is apparent that additional work dealing with X-agglutininogen is in order.

*Substance Responsible for Immunity and Neutralizing Antibody* That there is an antigenic substance or substances in active vaccine virus which stimulates the production of immunity and neutralizing antibody in convalescent human beings and animals has been established for many decades (44, 117). The nature of this substance in active virus is almost unknown. The subject suffers from the lack of crucial experiments which integrate the few known facts and also from the floods of reports which are frequently directly contradictory. Even such a basic observation as the production of immunity by injection of inactive virus is still a matter of controversy in the literature. This is true notwithstanding the fact that careful workers have adequately demonstrated in properly controlled experiments that the materials, in the forms used, are incapable of eliciting either significant degrees of immunity or appreciable amounts of neutralizing substances. Knowledge in this portion of the field has advanced little since the publication of complete reviews in recent years (13, 33, 122). The need for serious study on the material in vaccine virus responsible for the production of immunity is obvious.

One is also discouraged by the lack of understanding of the mode of action of neutralizing antibody. Craigie's comment in 1939 on reports dealing with the activity of neutralizing antibody *in vitro* is still pertinent. He said "the extremes of opinion regarding their interpretation being either that no *in vitro* reaction is possible between virus and neutralizing antibody or that an *in vitro* reaction does occur" (13). Perhaps workers who study vaccine virus should not be too severely criticized for their ignorance since the statement is equally applicable to the opinions of those who handle other viruses (certain plant viruses (8)).

and rabbit papilloma virus (47) excepted) Furthermore, the same general phenomena are also active in the neutralization of bacteria by antisera and here too the mechanism of the reaction is not completely understood (24, 34, 66) An encouraging approach to the problem has been made by Bronfenbrenner and his group who performed initial experiments with bacteria and subsequently applied their experience to bacteriophage (34, 35, 66)

*Summary of Immunological Reactions of Vaccinia* It is evident from the experimental data just cited that the virus of vaccinia is highly complex in its antigenic structure Four distinct antibodies are demonstrated in hyperimmune sera by *in vitro* techniques, *e g* L, S, NP and X, in addition, neutralizing substances are present in antisera and are detectable by *in vivo* experiments Neutralizing antibody is distinct from the L-, S-, and NP-specific immune substances and it may be unrelated to X-agglutinin Two of the antigens responsible for the stimulation of antibodies have been obtained from vaccine-infected tissue or from the virus itself in purified forms and their properties have been carefully studied, these are the LS-protein and the NP-nucleoprotein The nature of the antigenic substance or substances in active virus responsible for the appearance of X-agglutinin and neutralizing antibody in sera of immune animals is not known

The antigenic complexity of vaccine virus is not unique among viruses and resembles that generally found in the field of bacteriology A number of the adequately studied viral agents, *i e*, yellow fever, psittacosis, myxomatosis, influenza, bacteriophage and lymphocytic choriomeningitis, have been shown to give rise to more than one specific antibody (95, 120) Papillomatosis of rabbits and certain of the plant viruses are different in this respect since they elicit only a single specific antibody and it reacts with the virus *in vitro* and also neutralizes the virus *in vivo* (8, 47)

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# IMMUNITY IN BRUCELLOSIS<sup>1</sup>

I FOREST HUDDLESON

*Department of Bacteriology and Hygiene, Michigan State College, East Lansing, Michigan*

The principal purpose of this review is to bring together many of the data that are related directly or indirectly to the state of active immunity to brucellosis in animals and man, to critically analyze the data as to their significance, and to point out if possible the nature of the immunity. The term "active immunity" will be interpreted in this review with the same meaning so well expressed in the following definition given by Rivers (1941) "Active immunity is a state of resistance to infection engendered by a normal spontaneous attack of an infectious disease, by the experimental or intentional production of the disease or a modified form of it, or by the injection of vaccines." Data having a bearing on two possible methods by which an actively acquired immunity may be engendered will be reviewed. One is associated with recovery from natural or experimental infection and the other pertains to the injection of living or dead organisms, or their antigenic constituents. Since the only sure indicator of active immunity to brucellosis is resistance to infection, the data and observations bearing on the subject will be discussed with this objective in view.

## THE BOVINE

### *Active immunity acquired through infection*

Approximately 55 years have elapsed since bovine brucellosis was recognized as a disease entity and its study undertaken in a scientific manner. In view of the fact that the chief symptom of the disease in the cow is the premature expulsion of the fetus, Bang (1906) and many of those who followed devoted most of their efforts toward the symptom and its prevention rather than toward the diseased animal. Even diagnostic procedures were first studied with the object in view of determining whether an animal would abort or the abortion was due to *Brucella abortus*. Preventative measures and likewise active immunity were considered and studied from the standpoint of preventing the occurrence of the symptom. Bang, the master observer, permitted little to escape his attention and was not long in noting that many infected cows aborted only once. He speculated on the possibility of an immunity against abortion being acquired from infection.

Most of the essential data that have accumulated during the past 50 years and which serve as a basis for establishing proof that it is possible for cattle to acquire an immunity to brucellosis through infection were not collected and recorded with this object in view. Nevertheless, when the data are fitted together and considered in this direction, they furnish adequate proof that cattle very often acquire immunity to brucella infection.

The view has been expressed by many that bovine brucellosis is of the nature of a chronic disease and, since there are few, if any, examples of active immunity

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in infectious diseases of this character, one should not expect to find any active immunity to arise either after recovery or from vaccinal agents. Such asthenic reasoning would seem out of place in the twentieth century.

As early as 1916 data were collected by the writer from experiments on new-born calves which indicated that they were capable of resisting infection. The calves were exposed to infection either by nursing infected udders or by being fed live organisms added to milk. Most of the calves failed to produce demonstrable antibodies in the blood serum after exposure and, if they were present at the beginning of the feeding experiment, they disappeared within four to twelve weeks. No attempt was made at the time to recover brucella from the tissues of the calves. However, another study (1924) was conducted on 11 calves which had been exposed to infection during the nursing period, and all of which had shown serum antibodies for a short period. No evidence of persisting infection was found when the animals were slaughtered during the first pregnancy or after parturition. During the period of observation, 14 control animals housed on the same premises also remained free from infection.

In connection with the appearance of brucella antibodies in the blood serum of new-born calves, it was pointed out by Little and Orcutt (1922) that they are derived from colostrum containing such antibodies when ingested shortly after birth.

Carpenter (1924) added an important chapter to the story by demonstrating that brucella can be recovered from many organs of calves during and shortly after the feeding of milk containing the live organisms. The most interesting part of his finding was that the organisms disappeared from the tissues in the fifth week after the feeding was discontinued.

Quinlan (1923) made a study of 40 calves, 15 of which were infected dams, to determine their susceptibility to brucella infection by feeding them infected milk over a period of several weeks. Only 8 of the calves showed specific agglutinins in their blood, and then for a short time only during the period of observation, although many of them were kept under observation for as long as two years. These observations furnish additional proof of the resistance of calves to infection by brucella, and that the resistance during the exposure period does not necessarily come from the ingestion of colostrum containing specific antibodies.

Fitch and associates (1941) collected 56 female calves from brucella-infected dams to determine whether such calves, if infected, would carry the infection through to maturity and suddenly manifest evidence of the disease. The only known exposures to infection were at the time of birth and by way of infective milk during the first week of nursing. The group of animals was observed over a period of one to six parturitions. In no instance was there any evidence of infection continuing from birth or of its sudden appearance after these animals had become mature.

When one considers the results of all of the investigations together, there can be found few, if any, exceptions to the rule that calves up to a certain age possess a high degree of resistance to infection by brucella. Furthermore, the calf does not necessarily acquire its resistance toward infection as a result of ingesting

colostrum containing specific brucella antibodies. The calf from a non-infected cow, and not receiving colostrum high in brucella antibodies, appears to be just as resistant to infection up to a certain age as those that do.

There is one very important possible difference in the status of calves exposed and not exposed to infection that no one has yet thoroughly investigated. It is the difference in their susceptibility toward infection after they have reached breeding age. In conducting immunization experiments on young or mature cattle it would seem imperative, before declaring the efficacy of any immunizing agent, to know whether exposure during an early age had already left the animals with a high degree of immunity.

There came from one experiment conducted by Birch and Gilman (1925) an indication that calves, when exposed to infection at an early age, may not develop a sufficient immunity to protect them against an overwhelming experimental infection during adult life. In this particular experiment 9 pregnant heifers, 8 of which as calves had been in contact with infected animals, were exposed to infection three times a week for a period of three months by placing a suspension of *B. abortus* in the feed. Only one of the 8 resisted infection. The exception failed even to develop a high agglutination titer during the time of observation.

Although most young calves possess a high resistance to infection, it is well known that a very small percentage do become infected under natural conditions and remain so to maturity.

There may be no connection between the state which accounts for the resistance of young calves to brucella infection and that which is brought into action in adult animals as a result of exposure to infection. Two entirely different immunological phenomena may be involved. On the other hand, it is of interest to note that both resistant calves and adult animals do not develop serum antibodies unless exposed to or injected with massive doses of the organism. When antibodies do appear they remain for only a short time. This phenomenon appears to be associated with the presence of an active immunity to brucellosis in laboratory animals and human beings as well as in cattle, and will be pointed out in other parts of the review.

There is another period in the life of the bovine species when it manifests a high degree of resistance to brucella infection. This occurs during the period when the mature heifer and cow are non-pregnant. This fact was clearly demonstrated by Edington and Donham (1939) in a well-conducted series of experiments on pregnant and non-pregnant heifers and non-pregnant cows. The investigators exposed 12 pregnant and 15 non-pregnant heifers, and 6 non-pregnant cows to very large numbers of *B. abortus* organisms either by way of the eye, mouth, or vagina. In no instance was there any evidence to indicate that the organism had established itself in the non-pregnant animals. Of the 12 pregnant ones, 11 became infected and aborted. Seven of the heifers and the 6 cows exposed prior to breeding were again exposed to a large dose of *B. abortus* organisms during the subsequent period of gestation. Again, these animals failed to develop the disease. Although most of the animals developed a high agglutination

titer after exposure to the massive doses of organisms, there was a rapid decline in the titers in subsequent months

The failure of the pregnant animals to become infected after the second exposure can be attributed only to the operation of an active immunity resulting from exposure to live organisms when they were non-pregnant. It would be rare indeed for all members of such a large group of normal pregnant animals to resist infection by any other means. The low susceptibility of non-pregnant heifers and cows may not be of the nature of an acquired immunity, but it may be related to the same state which exists in young calves, and which protects them from infection,—a state which is unfavorable for the growth and persistence of the invading organisms.

It has been observed by many that the incidence of brucella infection appears to decrease after the disease has been present in a herd for many years, provided no additions are made to the herd from the outside. This occurrence has been attributed to "decreased virulence" of the organism in the animals in the herd. It would, however, be just as logical to assume that the decrease in the incidence of new infections was due to an actively acquired immunity resulting from exposure to infection at an early age. In this connection considerable data from experimental infection studies have been obtained that have a direct bearing on the occurrence of active immunity under natural conditions. Rettger and associates (1926) exposed 26 bred and unbred heifers to brucella infection by either the oral, subcutaneous or urethral route. Their protocols show that 12 (54 per cent) failed to become infected, and a large percentage failed even to develop specific serum antibodies. It is hardly conceivable that the animals failed to become infected because the number of organisms to which they were exposed was too small. A high degree of immunity must have been present in many of the animals before exposure, or was acquired immediately as a result of the exposure.

In an experiment conducted at the University of Wisconsin with the purpose of determining the influence of an adequate and low mineral intake in cattle on their susceptibility to brucellosis by artificial exposure, Hadley and Welsh (1931) obtained data indicating that an active immunity was operating in some of the animals used in the experiment. Of a total of 33 in the two groups, 13 failed to become infected. Many of the 13 developed specific serum agglutinins in low titer for only a short time. The animals were kept under observation for two years and during this time 29 (88 per cent) had ceased to show any evidence of being infected as indicated by the absence of specific serum agglutinins and by negative bacteriological findings. Later, Beach and Humphrey (1935) used 13 of these animals in an experiment to determine their resistance to infection, the outcome of which will be discussed later.

Huddleson and Smith (1931) studied a large number of animals in a dairy herd for a period of four to eight years and obtained data indicating the existence of active immunity in many to brucella infection. During this period, the serum agglutination test was positive in a dilution of 1:100 or higher in 189 animals. A considerable number of these aborted. There were many opportunities for all

# IMMUNITY IN BRUCELLOSIS

1933		1935	
Aug	Sept	Aug	Sept
1	2	3	4
5	6	7	8
9	10	11	12
13	14	15	16
17	18	19	20
21	22	23	24
25	26	27	28
29	30	31	32
33	34	35	36
37	38	39	40
41	42	43	44
45	46	47	48
49	50	51	52
53	54	55	56
57	58	59	60
61	62	63	64
65	66	67	68
69	70	71	72
73	74	75	76
77	78	79	80
81	82	83	84
85	86	87	88
89	90	91	92
93	94	95	96
97	98	99	100

COW NO	1933					1934						
	Aug	Sept	Oct.	Nov	Dec	Jan	Feb	Mar	April	May	June	July
4	---	---	---	---	---	---	---	---	---	---	---	---
38	PT	---	---	---	PP	PTT	PT	PT	PT	PT	PT	PT
44	PT	---	PT	---	TT	TT	TT	TT	TT	TT	TT	TT
28	PT	---	TT	---	PT	PT	PT	PT	PT	PT	PT	PT
40	PT	---	TT	PT	PT	PT	PT	PT	PT	PT	PT	PT
36	P	---	---	---	TT	TT	TT	TT	TT	TT	TT	TT
12	PT	---	---	---	TT	TT	TT	TT	TT	TT	TT	TT
8	---	---	---	---	PT	PT	PT	PT	PT	PT	PT	PT
17	T	---	---	---	TT	TT	TT	TT	TT	TT	TT	TT
5	PT	---	PT	PT	TT	TT	TT	TT	TT	TT	TT	TT
31	---	---	+++	+++	PT	PT	PT	PT	PT	PT	PT	PT
33	PT	---	---	---	TT	TT	TT	TT	TT	TT	TT	TT
35	PT	---	PT	PT	PT	PT	PT	PT	PT	PT	PT	PT

Dates of exposure Sept 5 and 15, 1933  
Serum dilutions 1 25, 1 50, 1 100, 1 200, 1 400 represented by consecutive -, +, P or T  
+ = complete agglutination, P = incomplete agglutination, T = trace  
\* Indicates calving date  
† Indicates aborting date

Dates of exposure	Serum dilutions	Sept 5 and 15, 1933	1 25 1 50 1 100	PT
-------------------	-----------------	---------------------	-----------------	----

Period of exposure	Sept 5 and 15, 1933
Serum dilutions	1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, 1:51200, 1:102400, 1:204800, 1:409600, 1:819200, 1:1638400, 1:3276800, 1:6553600, 1:13107200, 1:26214400, 1:52428800, 1:104857600, 1:209715200, 1:419430400, 1:838860800, 1:1677721600, 1:3355443200, 1:6710886400, 1:13421772800, 1:26843545600, 1:53687091200, 1:107374182400, 1:214748364800, 1:429496729600, 1:858993459200, 1:1717986918400, 1:3435973836800, 1:6871947673600, 1:13743895347200, 1:27487790694400, 1:54975581388800, 1:109951162777600, 1:219902325555200, 1:439804651110400, 1:879609302220800, 1:1759218604441600, 1:3518437208883200, 1:7036874417766400, 1:14073748835532800, 1:28147497671065600, 1:56294995342131200, 1:112589990684262400, 1:225179981368524800, 1:450359962737049600, 1:900719925474099200, 1:1801439850948198400, 1:3602879701896396800, 1:7205759403792793600, 1:14411518807585587200, 1:28823037615171174400, 1:57646075230342348800, 1:115292150460684697600, 1:230584300921369395200, 1:461168601842738790400, 1:922337203685477580800, 1:1844674407370955161600, 1:3689348814741910323200, 1:7378697629483820646400, 1:14757395258967641292800, 1:29514790517935282585600, 1:59029581035870565171200, 1:118059162071741130342400, 1:236118324143482260684800, 1:472236648286964521369600, 1:944473296573929042739200, 1:1888946593147858085478400, 1:3777893186295716170956800, 1:7555786372591432341913600, 1:15111572745182864683827200, 1:30223145490365729367654400, 1:60446290980731458735308800, 1:120892581961462917470617600, 1:241785163922925834941235200, 1:483570327845851669882470400, 1:967140655691703339764940800, 1:1934281311383406679529881600, 1:3868562622766813359059763200, 1:7737125245533626718119526400, 1:15474250491067253436239052800, 1:30948500982134506872478105600, 1:61897001964269013744956211200, 1:123794003928538027489912422400, 1:247588007857076054979824844800, 1:495176015714152109959649689600, 1:990352031428304219919299379200, 1:1980704062856608439838598758400, 1:3961408125713216879677197516800, 1:7922816251426433759354395033600, 1:15845632502852867518708790067200, 1:31691265005705735037417580134400, 1:63382530011411470074835160268800, 1:126765060022822940149670320537600, 1:253530120045645880299340641075200, 1:507060240091291760598681282150400, 1:1014120480182583521197362564300800, 1:2028240960365167042394725128601600, 1:4056481920730334084789450257203200, 1:8112963841460668169578900514406400, 1:16225927682921336339157801028812800, 1:32451855365842672678315602057625600, 1:64903710731685345356631204115251200, 1:129807421463370690713262408230502400, 1:259614842926741381426524816461004800, 1:519229685853482762853049632922009600, 1:1038459371706965525706099265844019200, 1:2076918743413931051412198531688038400, 1:4153837486827862102824397063376076800, 1:8307674973655724205648794126752153600, 1:16615349947311448411297588253504307200, 1:33230699894622896822595176507008614400, 1:66461399789245793645190353014017228800, 1:132922799578491587290380706028034457600, 1:265845599156983174580761412056068915200, 1:531691198313966349161522824112137830400, 1:1063382396627932698323045648224275660800, 1:2126764793255865396646091296448551321600, 1:4253529586511730793292182592897102643200, 1:8507059173023461586584365185794205286400, 1:17014118346046923173168730371588410572800, 1:34028236692093846346337460743176821145600, 1:68056473384187692692674921486353642291200, 1:136112946768375385385349842972707284582400, 1:272225893536750770770699685945414569164800, 1:544451787073501541541399371890829138329600, 1:1088903574147003083082798743781658276659200, 1:2177807148294006166165597487563316553318400, 1:4355614296588012332331194975126633106636800, 1:8711228593176024664662389950253266213273600, 1:17422457186352049329324779900506532426547200, 1:34844914372704098658649559801013064853094400, 1:69689828745408197317299119602026129706188800, 1:139379657490816394634598239204052259412377600, 1:278759314981632789269196478408104518824755200, 1:557518629963265578538392956816209037649510400, 1:11150372599265311570767859136

\* Indicates calving date

† Indicates aborting date

animals to become exposed to infective materials Of a total of 227 cows studied, 23 remained negative, and 19 never showed more than an incomplete reaction to the agglutination test in a 1:25 dilution In other words, the results of the agglutination test alone indicated that 41 (18 per cent) of the total were able through some means to resist infection during a long period of time Certainly the failure of such a large number of animals to become infected when every opportunity was provided, can hardly be accounted for except through the operation of an acquired immunity

Beach and Humphrey (1935) have contributed the most significant and important data of all on the question of an actively acquired immunity developing in animals after recovery from brucella infection Thirteen of the animals mentioned in the experiment of Hadley and Welsh (1931), after a period of 5 years during which time they had not again been exposed, were exposed to *B. abortus* by

TABLE 2

*Reacting animals reverting to incomplete and negative reactions over a period of 4 to 6 years*  
Huddleson and Smith (1931)

	AGGLUTINATION TITER			
	+1:25	+1:50	+1:100	+1:200 and 1:500
	Total numbers			
	26	32	24	165
Reverting to partial or trace in 1:25				
Number	8	10	3	9
Per cent	31	31	13	5.5
Reverting to negative				
Number	0	0	2	2
Per cent	0	0	8	1.2

way of the eye and mouth during pregnancy It may be seen from table 1, in which the results of subsequent serological tests are set forth, that only 1 of the 13 became infected Of 22 pregnant heifers used as controls and exposed at the same time, 11 became infected and aborted

The question is often asked, what trend does the agglutination reaction take over a period of years in animals that have been exposed to natural infection? Data obtained by Huddleson and Smith (1931) and set forth in table 2 from a study of animals in a large dairy herd over a period varying from three to eight years serve as a partial answer Of a total of 189 animals showing an agglutination titer of 1:100 or higher, only 16 (8.5 per cent) reverted to a negative or slight reaction Of 58 showing a reaction less than 1:100, 18 (31 per cent) either ceased to react or showed only slight reaction in a 1:25 dilution During the period, 26 of the total never developed a titer higher than 1:25

When one examines the trend of serum agglutination titers in animals experimentally infected a remarkable difference is found With three exceptions,

all the animals which Hadley and Welsh (1931) used in the nutritional experiment ceased to react to the agglutination test within two years after the initial exposure. Their data are summarized in table 3.

The identification of adult cattle immune to brucellosis by means other than determining their resistance to artificial or natural infection belongs to the future. There is at present no indication as to what the procedure will be. The measurement of bactericidal antibodies in the serum of animals resistant to the disease does not appear to be a hopeful procedure. Although Irwin and Ferguson (1938) claim to have obtained a reduction in numbers of *B. abortus* in their bactericidal experiments conducted with serum from immune cattle, no one has yet observed complete killing or reduction in virulence of brucella in the presence of blood from such animals.

In recent electrophoretic studies of the mobilities and concentrations of proteins in blood serums from normal and brucellosis-immune cattle, San Clemente

TABLE 3

*Comparison of reactor cows on basis of degree of reaction two years after first exposure to B. abortus*

Hadley and Welsh (1931)

	HIGH REACTORS (005 OR HIGHER)		LOW REACTORS (02 to 005)	
	Number	Per cent	Number	Per cent
Included in statistics	18	100	15	100
Aborted	14	78	4	27
Calved normally	4	22	11	73
Later became non-reactors	14	78	15	100
Became low reactors and remained so	0	0	0	0
Remained high reactors	4	22	0	0
Developed udder infection with <i>Bru- cella abortus</i>	5	28	0	0

and Huddleson (1942) obtained results which would indicate that the factor characterizing the "immune state" is not to be found in the blood serum proteins. No measurable differences were obtained between the serum proteins of immune and normal cattle.

#### *Active immunity in cattle acquired by use of vaccinal agents*

As in many other infectious diseases the early attempts toward producing immunity by means of vaccinal agents against bovine brucellosis were conducted empirically and with little knowledge of the natural course of the disease. Little consideration was given to the preparation of the vaccinal agents or the status toward infection of the animals that were injected. It would appear that most of the early attempts at vaccinal immunization of cattle against brucellosis were pointless and a waste of energy.

Those who attempted vaccinal immunization of cattle cannot be criticized too severely for the manner in which the experiments were conducted or for the con-

clusions that were drawn from the data. One must remember that well-controlled experiments on brucellosis in cattle are expensive and that considerable time must elapse before the results are available. In very few instances have investigators been supplied with the required facilities or funds for conducting well-planned and conclusive experiments. They have had to resort to the use of animals not primarily maintained for use in a disease experiment, or to the cooperation of farmers who were willing to "take a chance."

The chief objective in early studies of vaccinal immunization was the prevention of the chief symptom, premature expulsion of the fetus. Little or no consideration was given to the prevention of active infection. This objective was pursued even for many years after Schroeder and Cotton (1941) had demonstrated the presence of brucella in milk from udders of infected cows and its elimination therefrom for long periods of time.

Bang (1906) attempted vaccination of unbred sheep and cattle by means of live and toluol-killed bouillon cultures of *B. abortus*. In the experiments in which the animals received injections subcutaneously before conception, a high degree of protection against the symptom of abortion was noted. Of 15 sheep so treated and exposed by feeding infective material all failed to abort, while four unvaccinated controls aborted. Of 10 goats treated with the toluol-treated cultures and exposed, 7 aborted. One of 4 heifers treated with live cultures and 3 of 5 heifers treated with killed cultures and exposed to infection aborted. Three out of five unvaccinated controls aborted.

In the light of our present knowledge concerning the disease, the results of the vaccination experiments of Bang have little significance. The number of animals used was too small and, furthermore, protection against infection was not considered. Bang's work, however, apparently established a procedure and an index of results in vaccinal immunization which were to be followed for many years in all parts of the world. They are the injection of live cultures before conception, and measuring the results on the basis of the occurrence of abortion.

Stockman and McFadyean over a period of years practiced the injection of unbred animals in England with a massive dose of live *B. abortus* in order to produce protection against abortion. An attempt was also made to observe the immunizing value of killed organisms on animals after breeding. The results obtained in two herds after the use of the two agents are summarized in a report by Stockman (1914) in table 4.

When one attempts to evaluate these data, it must be remembered that the only status of the animals taken into consideration before treatment was whether they had previously aborted or were unbred. No thought was given to determining past or present infection even though the agglutination test was being used for that purpose during this period. In measuring the final results, the criterion for immunity in the treated animals was their failure to abort. The status of the animals as regards infection after treatment was not determined.

Zwick and associates (1920) in Germany made use of various agents in an attempt to prevent abortion in cattle. They employed live and killed organisms alone and each mixed with specific agglutinating serum. Each animal received

the culture from two agar slants suspended in physiological salt solution. It is stated that many of the treated animals had aborted one or more times before treatment. No attempt was made to determine whether either the treated animals and those used as controls were infected before or after the beginning of the study. Their data (table 5) show a considerably lower abortion rate in the animals treated with live cultures than in the controls. In view of the fact that the data were compiled from questionnaires and no apparent attempt was made to determine whether the animals were infected before or after treatment, the results are of doubtful significance.

Jensen (1921) was one of the first to study the efficacy of vaccinating non-pregnant heifers with a live culture to prevent abortion. The heifers were given

TABLE 4

*Summary of results of vaccinating cattle against brucellosis in England by Stockman (1914)*

METHOD	NUMBER OF ANIMALS OBSERVED	ABORTED	PER CENT
Treatment with live organisms	493	32	6.5
Treatment with killed organisms	110	23	21
Controls	432	101	23.3

TABLE 5

*Results of vaccinating cattle against brucellosis in Germany by Zwirk and associates (1920)*

AGENTS EMPLOYED	NUMBER TREATED	NUMBER ABORTED AFTER TREATMENT	PER CENT
Killed organisms	937	117	12.5
Killed organisms mixed with specific serum	157	20	12.7
Live organisms	482	28	5.8
Live organisms mixed with specific serum	57	3	5
Controls	1,356	245	18.1

from 2 to 3 injections before conception. The study was made on 447 vaccinated and 424 controls in different herds. During the 2 years following vaccination, 23.7 per cent of the treated and 36.8 per cent of the control animals aborted. Jensen was not impressed with the results of his experiment.

Schroeder (1922) in an early attempt to determine the value of vaccines in preventing abortion, injected 11 non-infected, unbred animals subcutaneously with a suspension of live *B. abortus*, supposedly virulent. Four others received repeated injections of killed organisms after conception. There were 8 controls. The animals were bred 60 days after treatment, and then all groups were exposed to infection by feeding infective material from an aborted fetus. Of the 11 treated with live organisms, 1 aborted. Of the 4 receiving killed organisms, 2 aborted. Seven of the 8 controls aborted. While the results of this small experiment indicate that the injection of live organisms before breeding had some

influence in preventing the manifestation of the symptom of the disease, there was no evidence obtained to indicate the absence of infection

In table 6 are compiled data gathered by Hadley (1921) after injecting a suspension of live brucella organisms into bred and unbred heifers and cows, some of which had previously aborted, the object was to determine the influence of the injection on the incidence of abortion. No mention was made of the status of the animals as regards infections before or after treatment. It may be noted that few controls were used. Even though the number of abortions is slightly greater in the controls than in those treated, it is obvious such data contribute little information on the value of vaccination in producing an active immunity against infection.

Smith and Little (1923), having been impressed with the reported favorable results of live-culture vaccination in non-pregnant animals in England, proceeded to put the theory to test on non-pregnant heifers and one small group of cows. Smith and Little employed whenever possible a newly isolated culture of *B. abortus* as a vaccine, injecting approximately 2 to 5 billion organisms

TABLE 6

*Results of vaccinating cattle against brucellosis in Wisconsin by Hadley (1921)*

STATUS OF ANIMALS STUDIED	NUMBER OF ANIMALS OBSERVED	NUMBER ABORTED	PER CENT
Unbred heifers	127	28	22
Unbred heifers, controls	24	8	33
Unbred cows	277	28	10
Bred cows	35	6	17
Bred and unbred cows, controls	66	20	30

intravenously or subcutaneously. The experiments were designed more for the purpose of comparing the effects of injecting live organisms into animals with natural exposure to infection. As there is no indication that those treated were again exposed to infection, it must be assumed that when infection occurred it was due to the live organisms injected. Apparently the control animals used in the experiment were exposed to infection from the injected ones, as no mention is made of artificial exposure. All the experiments with one exception, therefore, can hardly be considered as an effort to test the immunizing efficacy of live culture injections. The one exception is a group of 34 heifers treated with a killed vaccine and then exposed to infected animals in one of the control groups. The results obtained from the two methods of exposure are summarized in table 7.

It is interesting to note that the number of abortions in the groups treated with the live culture is considerably smaller than in the control groups. The latter were exposed evidently after they became pregnant. From what is known of the natural course of the disease today, such results are not surprising. There was not, however, a great deal of difference between the percentages that actually

became infected in the two groups. These results are rather surprising and do not agree with the data collected by Edington and Donham (1939).

The results obtained by Smith and Little are sufficiently convincing in demonstrating that the non-occurrence of abortion in inoculated animals is far from being a suitable criterion of freedom from infection, and that the practice of injecting live virulent organisms into non-pregnant heifers or cows with the idea of protecting them against infection is likely to result in more harm than benefit.

Hart and Traum (1925) (see table 7), injected non-infected and non-pregnant heifers and cows subcutaneously with a suspension of live virulent *B. abortus* for the purpose of determining its value as an immunizing agent against in-

TABLE 7

*Summary of results of three separate investigations on live and killed culture vaccination of cattle against brucellosis (first pregnancy observations)*

INVESTIGATORS	NUMBER OF ANIMALS	TYPE OF ANIMALS (UNBRED)	TYPE OF VACCINE	EXPOSURE	ABORTIONS		INFECTED*	
					Num-ber	Per-cent	Num-ber	Per-cent
Smith and Little (1923)	64†	Heifers	L§	None	8	13	23	36
	34†	Heifers	K§	To those treated	5	15	9	26
	153‡	Heifers		To those treated	46	30	61	40
Hart and Traum (1925)	20†	Heifers	L	Artificial	0	0	4	20
	10†	Heifers	L	None	0	0	0	0
	16†	Cows	L	None	0	0	10	63
	15‡	Heifers		Artificial	6	40	12	80
Lubbenhusen, <i>et al</i> (1926)	27†	Heifers	L	Natural	2	7	6	22
	33‡	Heifers		Natural	5	15	10	33

\* Revealed by examination of fetus or milk

† Treated

‡ Controls

§ L = live virulent, K = killed

fection and whether the vaccinal strain persisted in the animal body. The heifers received 220 billion and the cows 80 billion organisms. The heifers were exposed to infective materials by the oral route. After injection and breeding, the lactating cows and one group of heifers were not again exposed to infection. Although 4 of the 20 heifers treated were found infected after parturition, none aborted. Of the 15 control heifers exposed by the oral route, 6 aborted and 12 became infected. In a group of 10 heifers treated before breeding and not exposed, no abortions or infections occurred. The 16 lactating cows were injected more for the purpose of obtaining information of the localization of the injected organisms in the udder. Of these, 10 were found later to be eliminating *B. abortus* in the milk, some for a period as long as 6 months.

Lubbenhusen and associates (1926) (see table 7), selected 27 non-infected,

unbred heifers from a large herd of dairy cattle in which brucellosis had been present for several years to test the efficacy of vaccination with live culture in preventing abortion and infection. Thirty-three unbred heifers were selected from the same herd as controls. The heifers were injected with 20 to 60 ml of a suspension of live organisms from newly isolated cultures made up to a turbidity of 2 on the McFarland scale. The treated and control animals were permitted to associate freely with the naturally infected animals in the herd. During the first period of gestation, 4 abortions occurred in the treated animals, only 2 of which were due to brucella. However, bacteriological examinations made at the time of abortion or parturition revealed 6 (22 per cent) infected. During the first period of gestation, 7 of the 33 controls aborted, 5 of which were due to brucella. Ten (33 per cent) of these became infected.

Lubbenhusen extended the observations on many of the treated and control animals through a second and third period of gestation. At the termination of the experiment covering more than three years, 30 per cent of the treated and 42 per cent of the control animals had shown bacteriological evidence of infection on one or more examinations. Since the treated and control animals had been more or less in association with infected animals from birth, it is quite possible that many of them had developed a considerable degree of active immunity before the experiment was begun. This might easily account for the failure to infect 70 per cent of the treated and 58 per cent of the controls. There is no means of determining whether the treated animals in this experiment became infected from the injection of live organisms or from natural exposure. Since many of the non-pregnant animals used in the infection experiments by Smith and Little (1923) became infected as a result of inoculation it can be assumed that this must have occurred in Lubbenhusen's experiment.

Although the data obtained by Smith and Little, Hart and Traum, and Lubbenhusen demonstrate the futility of trying to obtain active immunity by injecting live virulent organisms into animals without at the same time producing infection, they do, however, reëmphasize the fact that a considerable percentage of non-pregnant heifers and cows possess a high degree of resistance to *B. abortus* infection by any route. Furthermore, for some unexplainable reason the number of abortions that occur after conception in the infected animals is considerably less than the number of actual infections. It would appear from the collected data that if infection is not established by the inoculation of live organisms into animals, most of them subsequently will be found highly resistant to infection. Such results can only mean that it is possible to produce active immunity against brucellosis. But the procedure must be one which will not leave the treated animals infected.

A smooth culture of *B. abortus* was found by the writer (1922) which when injected in large numbers failed to infect guinea pigs or pregnant cattle. This culture, used as a live vaccine (Giltner and associates, 1929), was injected subcutaneously into a large number of non-infected cattle in several herds from 1920 to 1927 to determine if it would cause an increase in resistance against brucellosis. The experiments from 1920 to 1926 were not controlled as no non-infected

controls were employed. The data furnished information only on the harmlessness of the vaccine. Of 293 non-infected animals treated and observed for 14 to 18 months, only 3.7 per cent aborted. In the same herds were 167 untreated animals which were supposedly infected according to the results of the agglutination test. During the same period of observation 25 per cent of these aborted. In 1926 and 1927 many negative control animals were left in the herds in which other non-infected animals were treated. Of the 722 treated, 5.4 per cent aborted during the 14-month period that followed. During the same time 8.1 per cent of 370 controls aborted. There were 644 infected animals in the same herds, but the incidence of abortion in these was also low, it being only 9.9 per cent. Infection had been present for a number of years previously in most of the herds used in 1926-1927. It is known that the number of abortions and new infections decrease in such herds after a period of 3 or 4 years. So it is not surprising to find such a low incidence of abortion in all groups during the year that followed this experiment. The results have no positive significance insofar as protection against infection or abortion are concerned. They do, however, demonstrate that it is possible to inject both pregnant and non-pregnant cattle with a live culture of very low virulence without harmful results. The only serious criticism of this type of vaccine at the time was that it produced specific serum agglutinins which persisted in some instances for more than one year. Their presence in the serum of animals as a result of injecting the vaccine made it difficult to detect actual infection by means of the agglutination test.

The culture used in the previously mentioned studies was dissociated in 1929 to the point where the injection of large numbers into an animal caused no agglutinin response. Then, a series of experiments was begun in cattle in private herds to determine its immunizing value. The experiments extended over a period of 6 years (Meyer and Huddleson, 1936). The results of this study are summarized in table 8. The vaccine was injected into calves, bred and unbred heifers and cows, which were negative to the agglutination test. From 40 to 50 per cent of the negative animals in most of the herds were left for controls.

One may note from the data that the incidence of infection and abortion was unusually low in all groups, that is, treated, controls and infected. Infected animals had been present in most of the herds studied for a number of years previously. In animals from herds of this type, experimental data derived from the employment of agents for immunizing or therapeutic purposes are likely to mislead one into believing that the agent itself was responsible for the favorable results. Fortunes have been made by producers of preventative and therapeutic agents at the expense of cattle breeders by knowingly or unknowingly taking advantage of the natural course of brucellosis in cattle.

When the vaccine made from the dissociated culture was injected into supposedly non-infected animals in herds shortly after the first appearance of the disease, the results which followed were a conclusive demonstration that such a vaccine gave little, if any, protection to animals against the disease.

In 1925 Buck (1930) began a series of experiments designed to determine the efficacy of suspensions of live cultures of *B. abortus* in producing sufficient active

immunity in calves to protect against infection when they become mature. Several cultures of different degrees of virulence were used in the experiments. The final results seemed to indicate that one culture, No. 19, of moderate virulence had possibilities. The 3 calves treated with this culture resisted infection on artificial exposure.

TABLE 8

*Summary of results from an attempt to immunize cattle against brucellosis with a live dissociated culture of B. abortus*

Meyer and Huddleson (1936)

YEAR	GROUP	NUMBER OF HERDS	NUMBER OF ANIMALS	ANIMALS			
				Aborting		Infected*	
				Number	Per cent	Number	Per cent
1930	Treated	22	592	13	2.1	70	11.8
	Controls		221	17	7.8	55	24.9
	Infected		218	38	17.4		
1931	Treated	22	611	23	3.7	89	14.6
	Controls		159	19	11.9	46	28.8
	Infected		227	50	22.0		
1932	Treated	27	592	10	1.7	31	5.2
	Controls		182	0	0	7	3.8
	Infected		235	36	15.3		
1933	Treated	27	481	6	1.2	30	6.2
	Controls		204	4	1.9	15	7.3
	Infected		213	29	13.6		
1934	Treated	19	397	12	3.0	34	8.5
	Controls		381	4	1.0	26	6.8
	Infected		170	18	10.5		
1937	Treated	14	504	4	0.7	6	1.1
	Controls		251	7	2.7	13	5.1
	Infected		182	29	25.8		

\* Based on bacteriological and serological data

The study begun by Buck was continued by Cotton, Buck and Smith (1934). The results of two experiments are summarized in table 9. In one experiment, 6 heifers near breeding age were treated with strain No. 19. In another, 9 were treated. After breeding they, along with controls, were exposed to infection by way of the conjunctiva. In the first experiment, 1 treated and all 8 controls became infected. In the second experiment, none of the treated, but 7 of 11 controls became infected. Two of the treated animals aborted, but no evidence of brucella infection could be found. The results of these studies led Cotton and his associates to suggest that calves between the ages of four and six months

be treated with strain No 19 in order to avoid a prolonged serum agglutination titer from the vaccine which would occur if adult animals were injected

Since 1938 there have appeared numerous reports (see table 9) pertaining to the efficacy of strain No 19 vaccine in the prevention of brucellosis in cattle in private and experimental herds Hardenbergh (1939) vaccinated 143 calves, leaving 73 controls They were maintained in a private herd under natural conditions of exposure Of the total treated, 3 (2 per cent) became infected after reaching maturity Four (6 per cent) of the controls became infected Haring (1938, 1939) Mills (1940), Thomsen (1939), Tompkins (1940), Haring and Traum (1937, 1941) and Winter (1941) have likewise vaccinated a large number of calves that were maintained under natural conditions All report encourag-

TABLE 9

*Summary of results from vaccination of calves with BAI strain No 19 as reported by various investigators*

ORIGIN OF STUDY	METHOD OF EXPOSURE	VACCINATED		CONTROLS	
		Total	Number became infected	Total	Number became infected
Cotton, <i>et al</i> (1934)	Artificial	15	1 ( 7%)	19	15 (78%)
Hardenbergh (1939)	Natural	143	3 ( 2%)	73	4 ( 6%)
Mills (1940)	Natural	142	12 ( 9%)	46	16 (34%)
Thomsen (1939)	Natural	266	9 ( 3%)	135	34 (26%)
Tompkins (1940)	Natural	24	4 (17%)	32	9 (28%)
Tompkins (1940)	Natural	222*	3 ( 1%)	0	
Birch, <i>et al</i> (1941)	Natural	35*	10 (29%)	23*	17 (74%)
Mohler, <i>et al</i> (1941)	Natural	8,182†	128 ( 1%)	0	
Haring and Traum (1941)	Natural	2,872‡	169 ( 5%)§	1,763	245 (13%)§
Winter (1941)	Natural	968*	63 ( 6%)	0	0
Rabstein and Welsh (1941)	Natural	642	5 ( 0%)§	0	0

\* First parturition

† Report covers part of 3 parturitions

‡ Number of pregnancies

§ Number of abortions

ing results on the efficacy of the vaccine in protecting cattle against infection after they reach breeding age

Birch, Gilman and Stone (1941) have conducted an exceptionally well-planned and controlled experiment on 35 calves with strain No 19 to determine its immunizing value and the duration of the immunity The animals, after breeding, were exposed to infection by placing them in quarters with aborting cows Of the 35 vaccinated animals and 23 controls, 3 of the former and 14 of the latter became definitely infected during the first pregnancy Ten of the vaccinated and 17 of the controls also became reactors Twenty-eight of the vaccinated and 14 of the controls were observed through a second pregnancy During this period 7 vaccinated and 5 controls became infected Seven in both groups became reactors

It is of interest to note that during the first period of this experiment 25 of the vaccinated and 6 of the control animals failed to develop specific agglutinins even though they were in constant association with infected animals. In a personal communication, Birch has informed the writer that 17 out of 18 vaccinated animals and all 6 of the controls that remained negative to the agglutination test through the first pregnancy also kept the same status through the second pregnancy. During a third pregnancy, 5 more of the 18 vaccinated animals became reactors. Of 5 remaining controls all remained negative. None of the animals in question aborted or showed *B abortus* in the milk. One might raise the question as to whether the animals in the control groups which failed to become infected were naturally immune or had already acquired an active immunity before exposure. Their failure to produce specific serum agglutinins on exposure is similar to what one observes in young calves and to the findings of Beach and Humphrey (1935) in cattle that had recovered from brucellosis. Perhaps the same immunological phenomenon is involved in both natural and actively acquired immunity to brucellosis.

The Bureau of Animal Industry of the U. S. Department of Agriculture has been conducting extensive studies of strain No. 19 in calves in privately owned herds since 1936. Mohler and associates (1941) have summarized the results in the following paragraphs:

"Of the calves vaccinated, 8,182 have now dropped calves involving three pregnancies, of which 5,673 were first, 2,026 were second, and 483 were third pregnancies.

"There were 7,782, or 96.2 per cent, normal parturitions in these herds. Of the latter number 6,526, or 82.9 per cent, calved normally and also were negative on post-parturition test, 399, or 5.1 per cent, calved normally but were positive to the post-parturition test, and 947, or 12 per cent, calved normally and were suspicious to the post-parturition test.

"On the other hand, 310 or 3.8 per cent abortions occurred in these groups, of which 182, or 58.7 per cent, of the aborting animals were negative to the post-parturition test and 99, or 31.9 per cent, were positive to this test, while 29, or 9.3 per cent, of the aborting animals were pronounced suspicious. Consequently, on the basis of the blood agglutination test, only 128, or 1.6 per cent of the abortions occurring in this group of 8,182 animals involved in the three pregnancies could be attributed to brucellosis."

Rabstein and Welsh (1941) have studied the effects of vaccination with strain 19 on animals from the standpoint of the persistence of the agglutinin response as well as the immunizing value. The study included three age groups of which 642 were vaccinated between 3 and 8 months (Group I), 89 between 9 and 12 (Group II), and 65 between 13 and 21 months of age (Group III). All of the 796 animals were positive to the serum agglutination test in a dilution of at least 1:200 two weeks following vaccination with the exception of two which remained negative even after being repeatedly vaccinated. Vaccinated calves showing a serum agglutination titer of at least 1:200 were in direct association with susceptible animals and in no instance did the latter show any change in their status.

There was noted a direct relationship between the age of the animal at the time of vaccination and the length of time that a positive blood reaction was retained. Nine months following vaccination, 91 per cent of Group I, 50.5

per cent of Group II, and 20 per cent of Group III were negative to the agglutination test. At 18 months following vaccination, Group I showed 14 per cent positive and 34 per cent suspicious, Group II contained 67 per cent positive and 213 per cent suspicious, while Group III had 20 per cent positive and 40 per cent suspicious.

Of the pregnancies recorded on animals vaccinated in this experiment, 172 had one calf, 90 had two, 48 had three, 26 had four, and 8 had five calves each. Out of the total number of pregnancies, ten (15 per cent) terminated in abortions of which five appeared to be due to *B. abortus* infection.

From the studies that have thus far been made with strain No. 19 as an immunizing agent against bovine brucellosis, it is reasonable to conclude that, when it is used on calves between the ages of 4 to 8 months, a high degree of active immunity is produced against natural brucellosis infection during the first pregnancy, that active immunity remains even during the second and third pregnancy, that the organism contained in the vaccine used on calves does not

TABLE 10

*Summary of a vaccination experiment on cattle conducted by McEwen in England (1937)*

YEAR	VACCINATED		CONTROLS	
	Number	Number became infected	Number	Number became infected
1st	109	4 (4%)	98	5 (5%)
2nd	90	2 (2%)	73	14 (19%)
3rd	38	0	29	7 (24%)

establish itself in the animal body to produce a carrier state, that calves and young heifers show an agglutinin titer for only a few months after vaccination.

The proper and continued use of strain No. 19 vaccine should serve a useful purpose in preventing the spread of brucellosis in infected herds and in preventing its occurrence in those herds free from brucellosis. It may play as useful a role in the control of brucellosis (Bang's disease) as the slaughtering of infected cattle.

McEwen (1937 to 1939) in England also has investigated the possibility of using a live culture of *B. abortus* of low virulence for immunizing adult animals as well as those under breeding age. In 1937 appeared the first comprehensive report of his studies in this direction, in which a sufficient number of controls were left to give the results significance. The data from one of McEwen's experiments are presented in table 10. There is little, if any difference between the incidence of infection in the vaccinated and control animals during the first year of observation. McEwen's explanation for this is that the herd employed contained many infected animals which reacted to the agglutination test and possibly many of those that were negative to the test were also infected. If the latter were injected with a vaccinal agent, they would later show evidence of infection and be classified as unprotected by the vaccine.

During the second year that the animals were under observation in McEwen's experiment there was a marked difference between the number that became infected in the treated and in the control groups

*Active immunity in the goat acquired through infection or by use of vaccinal agents*

Although the milch goat has been known to be the host and disseminator of *Brucella melitensis* in certain regions since 1905, there are not available any recorded data of either a positive or negative nature pertaining to recovery from the disease and the status of such goats toward a second infection after recovery. The lack of studies in this direction on the goat might appear paradoxical to those not acquainted with the almost insurmountable difficulties which have confronted those who have attempted to study the disease. The type of milch goat inhabiting the enzootic regions has never been considered of great economic importance, except perhaps to the owner. When to this view is added a poorly organized and inadequately supported animal health service in the same regions, it is not surprising to find that the course of brucellosis in the goat has not yet been explored.

The infected goat continues to be the chief source of brucellosis in human beings throughout the world. But until more information is available concerning the nature of the disease in this host, one cannot expect any considerable reduction in the incidence of the disease in man.

The same situations that have obstructed studies of the infected goat since the Mediterranean Fever Commission was disbanded also have delayed until 1937 any well-directed efforts toward the study of vaccinal agents as a means of producing an active immunity in this animal. In this year Poulding (1939) began a series of experiments in Malta with this object in view. One involved the injection of a suspension of live virulent *B. abortus* into female kids which had been raised from non-infected females. When they had reached the age of 4½ to 5½ months, 12 kids were injected subcutaneously with 2,500 million live organisms. None of the kids became permanently infected from the inoculation as indicated by the disappearance of specific serum agglutinins within 8 months. They were bred on reaching maturity and shortly afterwards, together with 12 controls, exposed to *B. melitensis* infection by permitting them to associate in the same pen with infected female goats that had just aborted. Of the 12 treated goats, one aborted and one failed to conceive. Six of the controls aborted and one failed to conceive. At the time of parturition or abortion, 8 of the treated animals and 8 controls were found infected with *B. melitensis*.

Poulding also studied the immunizing value of another agent on goats. This was a bacteria-free culture filtrate prepared by growing *B. melitensis* in liver broth for a long period of time, then passing the broth through a filter and adding phenol to 0.5 per cent (this agent was reported on previously by Zammitt and Debono (1933)). Two groups of goats were treated with this agent. One group consisted of 15 one-year-old females, and the other of 14 females more than 2 years old the history of which was indefinite. After breeding the two groups they, along with 12 control females, were exposed to infected goats at the time

of abortion. Examinations made on the goats after abortion or after parturition revealed 11 of the 12 controls and 13 of the 29 treated ones infected. Only 2 in the group of older goats became infected.

The results of Poulding's experiments do not indicate that the injection of goats with live *B. abortus* or with a culture filtrate prepared from *B. melitensis* are of any value in actively immunizing them against *B. melitensis* infection. The prevention of the disease in goats is of sufficient importance to warrant continued study in this direction.

*Active immunity in the guinea pig and other laboratory animals acquired by use of vaccinal agents*

The guinea pig is perhaps the most susceptible small animal of all to experimental infection with any of the three species of *Brucella*. It is known that not more than 25 live *B. abortus* organisms, and 5 or less of *B. suis* when injected subcutaneously, will produce extensive gross changes in the guinea pig tissues within a four-week period of incubation.

In view of its susceptibility, the guinea pig affords an ideal preliminary testing ground for immunizing agents intended for use on large animals and humans. However, if an agent should fail to immunize guinea pigs against infection this does not necessarily imply that it will likewise prove ineffective when used on larger animals. On the other hand, if an agent confers on guinea pigs a high degree of active immunity, there are precedents for expecting the same to obtain in larger animals when it is used in a suitable dose.

For some reason, the guinea pig has been greatly neglected as a test animal for brucella vaccinal agents as indicated by the small number of published reports. Ascoli was the first to attempt the immunization of guinea pigs against *B. abortus* infection by the injection of heat-killed cultures of the same organism. His results were negative. Similar experiments with the same object in view were conducted by Stafseth (1920), Hagan (1922), Schroeder and Cotton (1924), Gwatkin (1933), and Holth (1933). All of these workers also failed to produce an active immunity in the guinea pig against *B. abortus* infection.

McEwen and Roberts (1936) have made the most thorough and comprehensive investigation of all to determine the value of killed and live avirulent culture vaccines for immunizing the guinea pig against *B. abortus* infection. Three types of vaccine were studied, (1) a suspension of live organisms prepared from a culture of *B. abortus* that was relatively non-infectious for guinea pigs, (2) a heat-killed suspension, and (3) a formalin-killed suspension of the same culture. Different groups of guinea pigs were injected with single and multiple doses of vaccine, and were exposed to infection at varying intervals after treatment along with normal controls. The investigators were unable to produce active immunity with the killed vaccines. Their protocols with respect to the immunizing efficacy of the live vaccine contain very conflicting data. In certain experiments, 90 per cent of the treated animals failed to become infected, whereas all controls were infected. In other experiments performed in a similar manner, little, if any difference was found between the incidence of infection in the

treated and controls McEwen and Roberts considered that loss of the immunogenic antigen in the culture after long cultivation on media might be the contributing factor to the variation in results. An experiment was performed to test this hypothesis by passing the culture through guinea pigs and then comparing its immunizing value with one that had been maintained on culture media. In this experiment 1 of the 10 guinea pigs treated with the animal passage culture became infected on exposure as compared with 4 out of 10 treated with the stock culture. Nine of 10 controls exposed at the same time become infected. The results of this experiment would indicate that loss of immunogenic property of the culture was largely responsible for the variation of results in the previous experiments.

Pennell and Huddleson (1937, 1941) and Stahl, Pennell and Huddleson (1939) have used the guinea pig in determining the immunizing value of such brucella agents as heat-killed organisms, live organisms of an R type, the protein nucleate fraction of the cell, a fraction derived from the cells by treatment with trypsin and trichloroacetic acid and by trichloroacetic alone. All of these agents failed to protect guinea pigs against experimental infection. A purified and concentrated brucella antiserum was prepared by Huddleson and Pennell (1939) which protected guinea pigs against the toxic effects of the toxic fraction by brucella but failed to protect them against infection. Kolmer, *et al* (1939) also succeeded in protecting mice against the toxic effects of large doses of brucella organisms through the injection of an antiserum prepared in rabbits, but they report no evidence which would indicate that the mice were protected against infection. Roman (1938) and Renoux (1939) prepared a soluble antigen from *B. melitensis* according to a procedure described by Boivin and Mesrobian (1934) and claim to have successfully immunized guinea pigs against *B. melitensis* infection. They found it necessary to inject the soluble antigen along with a live avirulent culture of *B. abortus* in order to obtain a high degree of immunity and, then, only in male guinea pigs. Stahl and Hamann (1941) were unable to confirm the results obtained by Roman or Renoux by preparing a soluble antigen in the same manner and using it in the same dosage in guinea pigs.

Since no one has succeeded in establishing an active immunity against brucella infection in either cattle or guinea pigs by injecting killed organisms, this would indicate that either a mild degree of infection must be produced in the animal before this state is attained, or that the immunizing antigen in the cells is extremely labile and will not withstand the action of physical or chemical agents.

Recently the writer (1942) has succeeded in obtaining from live brucella cells a water-soluble immunizing antigen, and from its study obtained sufficient data to prove the labile antigen hypothesis.

The water-soluble antigen is obtained from live wet cells of either *B. abortus* or *B. suis* by crushing them in a Booth and Green (1938) bacterial crushing mill and removing the crushed cell fragments by centrifugation at high speed. Ethyl ether is added to the supernatant to the point of saturation as a preservative and to kill any live organisms that might not have been removed.

During a period of one and one-half years a total of 247 male and female

guinea pigs, divided into 25 groups, were treated with 0.5, 1 and 2 mg amounts (dry weight basis) of the fraction, each pig receiving the respective dose at intervals of three days. From 15 to 25 days after the last dose all pigs in each group, along with an equal number of controls, were injected subcutaneously with live virulent *B. abortus* in numbers varying from 152 to 3,840. After a period of 4 weeks, the pigs were killed, the internal organs examined for gross evidence of infection, the blood serum tested for specific agglutinins and the tissues cultured for brucella. Of the total number treated only 9 per cent were found infected at the time of necropsy. Of 238 guinea pigs used as controls in the 25 experiments, 151 (63 per cent) were found infected.

The treated guinea pigs not only failed to show the organism in cultures taken from the tissues, but were free from gross tissue changes as well. Specific agglutinins were seldom, if ever found in the blood serum of those free from infection.

Before any degree of immunity could be obtained against experimental *B. suis* infection, using a fraction obtained from *B. suis*, it was necessary to inject guinea pigs with 3 successive 5 mg amounts at intervals of 3 days.

The component in the crushed-cell soluble material that is essential for producing an active immunity is easily destroyed by most antiseptics and by heat. Phenol in a final dilution of 1:200 and merthiolate in a final dilution of 1:10,000 in the material renders it inactive. So also does an exposure to a water-bath temperature of 56°C for 30 minutes.

The results of this study have furnished proof that an active immunity against brucella infection can be obtained without the intervention of the live organism and that the essential immunizing antigen in the cell possesses labile characteristics.

#### THE HUMAN BEING

##### *Active immunity acquired through infection*

In most of the studies of human brucellosis more emphasis has been placed on the clinical cases, their detection, and attempts at treatment rather than on a study of those that fail to show clinical manifestations of the disease even though the results of various diagnostic tests furnish positive proof that infection once existed. The golden opportunity to accumulate facts relating to the nature and occurrence of acquired immunity, that was missed from failure to study cases after recovery from infection whenever they occurred in animals, has again been presented and lost when similar cases and groups were found in human beings.

Proof of an acquired immunity to brucellosis developing in human beings after a clinical or sub-clinical manifestation of the disease is based largely on observations of specific serum and allergic tests and the interpretation given to the findings. The available data are of sufficient extent to be highly significant if they are interpreted by deductive analysis in terms of acquired immunity.

The laboratory diagnostic procedures which have been most widely used and which furnish the most reliable information toward confirming a diagnosis of

human brucellosis are the blood culture, the serum agglutination, the whole blood opsonic, and the skin sensitivity tests. The results of the last three also have been employed in obtaining valuable information relative to previous clinical and sub-clinical infection. The opsonic test, when properly performed is a valuable aid to the interpretation of a positive agglutination or skin test. It was pointed out by Huddleson, Johnson and Hamann (1933) that when most of the neutrophiles in citrated whole blood of a normal individual show a marked ingestion capacity for brucella cells, this finding usually signifies a previous infection and a high degree of resistance on the part of the individual in question to subsequent infection. That there are exceptions to this interpretation is now well known. That is to say, the blood leucocytes of a small percentage of clinical cases will also show a high degree of phagocytosis and likewise the leucocytes of a small percentage of those who have long since recovered will show a low ingestion power.

It may be the contention of some that the presence of demonstrable brucella antibodies in the blood serum or the presence of specific skin sensitivity to brucella allergens in a healthy individual, with or without a history of clinical manifestations of the disease, constitutes evidence of continued infection. There is, of course, a logical basis for their reasoning, which is that both acute and ambulant cases show the same detectable serum antibodies or skin sensitivity reactions. But when the same type of reactions can be induced in human beings by the injection of the proper agents without producing the disease itself, is it not logical to believe that after the live organism enters the body it could also induce specific serum antibodies and skin sensitivity and then be destroyed with signs of little, if any, clinical manifestation of infection? The writer does not wish to imply that the commonly known brucella serum antibodies and skin sensitivity, when found in a normal individual, necessarily play an important role in active immunity. At the present time they can only be interpreted as some of the signs which constitute proof that the body tissues of an individual have reacted to the live organism, and, when present after recovery from the disease or in the absence of any known history of the disease, indicate active immunity.

Considerable data have been accumulating for a number of years from the use of the specific tests on large groups of human beings. The results when carefully analyzed point in the direction of their significance. There is also circumstantial evidence of an epidemiological nature that can only be interpreted as proof that immunity to brucellosis is acquired by many individuals from exposure to the organism.

Let us first examine the circumstantial evidence. In doing so, it is realized that there are many who will view such evidence with skepticism, as unworthy of scientific consideration. Nevertheless, many important precedents can be cited in the history of medicine for not ignoring in this instance the circumstantial evidence pointing to the existence of an acquired immunity to brucellosis.

Since the development of accurate diagnosis procedures, from 200 to 500 cases of brucellosis have been diagnosed in the inhabitants of Malta each year, the

yearly population averaging approximately 220,000 over the past 50 years. Since it is known that the disease on the Island is acquired from drinking raw goat's milk or eating products from such milk, that approximately 15 per cent of the goats are infected with *B melitensis* and that, up to 1938, 90 per cent of the people consumed the milk in the raw state, it must be admitted that the number who are exposed each year, and year after year without showing clinical manifestations, is far greater than the number who do. This extreme difference in the number exposed and in the number known to become clinically infected, instead of being considered in its proper light, has been used by prominent individuals, not only in Malta but in other countries as well, as an argument against infective milk being one of the chief means of conveying brucellosis to human beings.

Since *B abortus* and *B suis* and their respective hosts, the cow and the hog, have become implicated in the occurrence of human brucellosis in the United States and other countries, the same situation as regards wide differences in the number exposed to those clinically infected has been apparent. And as in Malta, individuals have used this widely acknowledged situation to bolster the thesis that the consumption of animal products containing either *B abortus* or *B suis* is not the chief source of human brucellosis. Fortunately, there exist data from several surveys and studies on the human being with respect to *B abortus* and *B suis* which, when added together and analyzed, supply evidence that more infections have occurred than are apparent. Most of them were undoubtedly of a sub-clinical nature and would not have been detected without the use of specific tests applied to the blood serum or in the skin. That these individuals acquired a substantial immunity to the disease after the initial infection is borne out by their subsequent histories.

If one undertook the task of obtaining information that would aid in explaining why many individuals fail to develop the disease even though they are exposed repeatedly to infective materials, he would first examine those in occupations or professions who come in contact more often than others with brucellosis-infected animals or materials. The results of such examinations are well illustrated in the five following investigations.

Huddleson and Johnson (1930) were among the first to collect enlightening data on such a group, which served as the initial answer to the question of the occurrence of acquired immunity to brucellosis in human beings. They examined the blood of 49 practicing veterinarians for brucella agglutinins and questioned the individuals as to any past history of clinical manifestations of the disease. Of the total number examined, 28 (57 per cent) showed serum agglutinins in a titer of 1:50 or above. Only 3 of these gave a past history of a symptom-complex characteristic of the disease. From many of the veterinarians who showed serum agglutinins, it was learned that small eruptions appeared on the skin of the arm used in removing retained placenta from cows that had aborted. At the same time there also occurred a general malaise not unlike the symptoms of the disease. That these reactions were manifestations of a brucella allergy was proved by the injection of a specific allergen into the skin of the same

individuals and producing the same reactions. The presence of serum antibodies and skin sensitivity in such a high percentage of the individuals examined furnished indirect proof that they were at one time infected with brucella. Their failure to show clinical evidence of the disease, even though exposed repeatedly, can only mean that the initial infection was of a slight nature and caused the development of a high degree of acquired immunity.

Additional data, which showed that a large percentage of veterinarians develop signs of sub-clinical infection on exposure to infective materials and subsequently are never subjects of the disease, were obtained by Thomsen (1931) in Denmark. He found that 94 per cent of all veterinarians who had been in cattle practice for more than one year, showed specific antibodies for brucella in the blood serum, and a high percentage in practice for several years also show brucella skin allergy after removing retained placentas from infected cows. Thomsen believed that

TABLE 11

*Comparison of the results of the agglutination test with results of other laboratory tests on 41 clinical and 49 subclinical cases of brucellosis*

Huddleson and Munger (1940)

MAXIMUM AGGLUTINATION	41 CLINICAL CASES				49 SUB-CLINICAL CASES			
	Neg	1 25	1 50	1 100 or higher	Neg	1 25	1 50	1 100 or higher
Titers (no )	4	12	7	18	26	8	6	9
Blood culture, positive	3	12	7	13	1	0	1	1
Brucellergen test, negative	0	0	0	0	1	1	1	0
Brucellergen test, positive	4	12	7	18	25	7	5	9
Phagocytosis, low or moderate	3	10	6	14	16	6	4	7
Phagocytosis, marked	1	2	1	4	0	1	1	2
Phagocytosis, negative	0	0	0	0	10	1	1	0

the results of the test in the absence of the disease indicated the existence of an acquired immunity which came from exposure to infective material after entering practice.

Huddleson and Munger (1940) examined a total of 349 college students during a small epidemic of brucellosis due to *B. melitensis* for possible evidence of exposure to infection. Four tests were employed, namely, the agglutination, opsonic, skin sensitivity, and blood culture to determine the occurrence of clinical or sub-clinical infection. Not all those examined had had the same opportunity to become exposed. The number, therefore, that showed a positive reaction to one or more of the tests cannot be statistically analyzed in terms of the entire group. Of those examined, 41 were definite clinical cases. There were also 49 others in whom one or more of the tests indicated infection, but who continued to remain symptom-free. These data (see table 11) indicate that when a group of individuals is equally exposed to brucella infective material, there will be found among them as many who show evidence of infection without clinical manifestations as those who do.

Dooley (1932) was prompted to examine the blood serums of 263 boys in a boys' school for brucella agglutinins after two of them developed clinical brucellosis due to *B. abortus*. Of the total number examined, 41 per cent were found to show agglutinins in titers ranging from 1:40 to 1:12,000. All the boys, with two exceptions, remained in good health during a one-year observation period.

Wilson (1932) examined two groups of veterinarians, one of which had had ample opportunity to be exposed to *B. abortus* by contact with infected animals, while the other group had no history of such contacts. The agglutination test

TABLE 12

*Results of specific brucella tests on groups of humans obtained by various investigators*

OBSERVER	GROUP OBSERVED	LOCATION	TEST EMPLOYED					
			Agglutination		Opsonic		Skin allergy	
			Number tested	Per cent positive	Number tested	Per cent positive	Number tested	Per cent positive
Meyer, <i>et al</i> (1934)	V*	California			100	74	58	60
Huddleson, <i>et al</i> (1937)	V	Michigan	49	57	20	95	50	20
Lerche and Roth (1933)	V	Germany					44	93
Dubois and Sollier (1931)	V	France					14	29
Jordan (1931)	V	Iowa	120	45				
Kitelman (1932)	V	Kansas	88	30				
Meyer, <i>et al</i> (1934)	P*	California			161	67	615	50.6
Huddleson, <i>et al</i> (1933)	P	Michigan	167	11	167	24		
Jordan (1931)	P	Iowa	220	31				
Heathman (1934)	P	Minnesota	1,096	6.9			1,096	55.0
Lentze (1930)	F*	Germany	57	23				
Makkawejsky, <i>et al</i> (1931)	F	Russia	354	7.6				
Meyer, <i>et al</i> (1934)	F	California			30	57		
Meyer, <i>et al</i> (1934)	C*	California			103	35	54	11
Levin (1930)	C	Oregon					269	3
Huddleson, <i>et al</i> (1933)	C	Michigan					240	12.5
Huddleson, <i>et al</i> (1937)	C	Michigan	8,124	1.2	8,124	8.0	8,124	10.3
Keller, <i>et al</i> (1938)	C	Tennessee	1,247	2.1			1,247	4.8
Angle, <i>et al</i> (1938)	S	Missouri					7,122	8.7

\* V = veterinarians, P = packing house employees, F = farmers, C = clinic and hospital patients, S = school children

made on the blood serums of 63 of the former revealed 24 per cent showing a positive reaction, while in the latter group of 35 only 3 per cent showed specific serum agglutinins. Little, if any, positive evidence was obtained that those who reacted to the agglutination test had at one time been clinically infected with brucella.

Since 1930 there have been many investigations of a survey nature conducted on many groups of people by use of specific serum and allergy detecting tests chiefly to learn more about their significance and usefulness in detecting those who were once infected with brucella. The results that have been obtained in

a few of the surveys made on groups of human beings through the application of one or more of the specific tests are summarized in table 12. The groups are representative of those whose occupations bring them in close contact with hosts of brucella and those in the general population. The results of the tests clearly demonstrate that the greater the opportunity for exposure to infection, the larger will be the number of individuals who will show evidence of having been infected. The small percentage of positive reactions found by the agglutination test in comparison to the higher percentage obtained by the skin test is highly significant evidence that the majority had passed through the infection stage long before the tests were made. From the studies that have been made on many groups of people it has been shown that only a few of those, who have long since recovered from infection and who continue to show skin sensitivity, will show serum agglutinins in a titer of 1:20 or above. And as in the case of large animals, such individuals seldom, if ever show clinical evidence of infection or a considerable increase in serum agglutinins on repeated exposure to natural infection.

The proof that groups such as veterinarians, certain packing-house employees, farmers and many laboratory workers, who are free from clinical evidence of infection, but react to one or more of the brucella tests, are actively immune to the disease rests largely on the evidence that has been adduced from their histories. It is well known that an individual, who shows by the negative character of all tests no indication of ever having been infected, cannot long work with infected animals or infective materials without showing some evidence of either clinical or sub-clinical infection. So, when groups of people such as veterinarians continue to work year after year in the midst of infective materials without developing the disease, it is clear evidence that the initial infection, after its termination, confers upon the individual a high degree of immunity. It is true that such persons may show an allergic reaction, the symptoms of which closely resemble the disease, on exposure to the organisms or the protein of the organism by way of the skin, digestive or olfactory tract. The allergic reactions are seen more often in veterinarians and certain laboratory workers than in other groups and have been time and again mistaken for symptoms of the disease.

When one carefully analyzes all the data that have been collected in the numerous surveys on a large number of people by use of the specific tests in terms of their histories, the evidence in favor of the occurrence of an acquired immunity to brucellosis after clinical or sub-clinical infection is overwhelming. The only tests thus far employed that have the semblance of indicators of the immune state are the skin sensitivity and opsonic tests. Since the infected also show skin sensitivity and opsonins it is obvious that these tests alone cannot always be relied upon as criteria of active immunity. The real indicator of active immunity to brucellosis in the case of the human still awaits discovery.

#### *Active immunity in man acquired by use of vaccinal agents*

The many attempts that have been made at vaccinal immunization of human beings against brucellosis are good examples of wasted effort and stabs in the dark. The attempts have centered around the use of suspensions of one or

more of the species of *Brucella*, killed by heat or chemical agents. Instead of employing suitable controls in order to gauge the effectiveness of the vaccine, the investigators drew a comparison between the incidence of the disease in similar occupational groups before and after treatment. In view of what is known about the occurrence of sub-clinical or latent infections, it does not seem possible that anyone would attempt to evaluate the efficacy of a vaccinal agent from data obtained in this manner.

One of the most recent studies of a heat-killed vaccine involving a large number of people is that of Dubois and Sollier (1938). They treated with a heat-killed vaccine 4,022 persons whose occupations exposed them to infected animals or infective materials from such animals. Only two cases of infection occurred in the entire group during a period of observation varying from 1 to 8 years after treatment. Meyer and associates (1934), on the other hand, have presented the history of one individual who failed to receive any protection whatsoever from three injections of a heat-killed vaccine. They state emphatically that there is no experimental proof that vaccines prepared from killed bacteria are capable of conferring the least degree of protection against brucellosis. It may, therefore, be said that there is as yet no safe and effective vaccine for human use. A suitable one would occupy an important place in the prevention of the disease in those in hazardous occupations and in the general population of countries where sanitary practices meet with almost insurmountable obstacles.

#### RECAPITULATION

A critical analysis of the data now available pertaining to immunity in brucellosis furnishes convincing proof that such a state can be demonstrated in animals and man. In the bovine there appear to be two types of immunity, (1) a so-called "natural immunity" that functions in young animals up to or near the beginning of ovulation, (2) an acquired immunity usually developing in unbred heifers and cows and requiring for its stimulus the presence of live organisms in the body or the injection of a labile immunogenic constituent of the bacterial cell.

When virulent organisms enter the body of young animals under natural conditions or are injected in small numbers, they remain for only a short time and cause little, if any, reaction on the part of the tissues. Only when large numbers of organisms are injected is sufficient reaction provoked to cause the appearance of serum antibodies in a high titer, and even then such antibodies persist for only a short time.

A large percentage of normal non-pregnant heifers and cows, when exposed to brucella infective material instead of becoming actively infected, develop a high degree of active immunity against subsequent exposures. From the small amount of data now available it would appear that the immunity acquired through the infective process persists for more than 5 years.

When human beings are exposed to brucella, a large percentage of those exposed fail to show any clinical evidence of the disease, but do develop specific serum antibodies and skin sensitivity, and in some instances to the same degree as those clinically infected.

When antibodies or skin sensitivity are found present in healthy

state of active immunity is indicated. No convincing data have yet appeared pertaining to the presence of active immunity in persons who fail to show a reaction to one or more of the specific tests.

Although the demonstration of specific antibodies in the blood serum or specific skin sensitivity in normal human beings cannot be considered as the final and always certain means of detecting those who are actively immune to brucellosis, they will have to be accepted and utilized for what they are worth until the real indicator of active immunity can be discovered.

In the light of our present knowledge it appears that the commonly known brucella serum antibodies are by-products arising from the reaction between antigen and body tissues, and have no proved function in the active immunity mechanism.

From the facts now available it may be stated categorically that the possibility of animals and human beings acquiring an active immunity to brucellosis through infection is no longer a hypothesis but a demonstrated fact.

There is now conclusive proof that an active immunity can be established in adult cattle against brucellosis by injecting them as calves with live *B. abortus*, the vaccine being prepared from a culture of low virulence. The duration of the immunity produced in this manner has not been determined conclusively.

Although it seems highly probable that human beings could be immunized against brucellosis, there is up to the present time no convincing proof that an agent for this purpose has been developed.

The possibility of an active immunity occurring in swine to *B. suis*, the infecting species, has not been considered in this review due to the fact that no information is available. Very little is known even about the course of the disease under natural conditions. It has long been recognized that swine brucellosis is an important economic disease, and that the infected hog is a reservoir from which the disease is acquired by human beings and other animals. The well-planned research program on swine brucellosis now being undertaken at federal and state experiment stations, if continued, should furnish information on the course of the disease and its immunological aspects that is now so badly needed.

Since there are three known species of *Brucella*, and each may infect species of animals other than the one in which each is commonly found, it would seem essential to the successful application of measures to control the disease to know whether recovery from an attack of one species of *Brucella*, or vaccinal immunization with one species will afford protection against the other species. In the case of human beings, it has been observed repeatedly in many laboratories that those who have developed an immunity as a result of a clinical or sub-clinical infection with one species, may freely work with the others without becoming infected. This observation has been made so many times in several laboratories that it now seems certain that an immunity which is produced by one species will protect against an infection by the other two. There are also experimental data which show that the guinea pig can be immunized against *B. abortus* infection by treatment with the crushed cell fraction derived from *B. suis* as well as from *B. abortus*.

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# THE CYTOPHAGA GROUP A CONTRIBUTION TO THE BIOLOGY OF MYXOBACTERIA

R. Y. STANIER<sup>1</sup>

*Hopkins Marine Station of Stanford University, Pacific Grove, California*

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*Quand on a formé dans sa tête un de ces systèmes qui demandent à être vérifiés par l'expérience, il ne faut ni s'y attacher opiniâtrément, ni l'abandonner avec légèreté. On pense quelquefois de ses conjectures qu'elles sont fausses, quand on n'a pas pris les mesures convenables pour les trouver vraies. A force de multiplier les essais, si l'on ne rencontre pas ce que l'on cherche, il peut arriver qu'on rencontre mieux. Jamais le temps qu'on emploie à interroger la nature n'est entièrement perdu —De l'Interprétation de la Nature, DENIS DIDEROT, 1754*

If one examines the developmental history of botany and zoology, three major epochs become apparent. During the first, the natural historical period, plants and animals were subjected to a broad survey through which their salient characters and general conditions of existence were elucidated. This merged almost imperceptibly into the second period of systematization, which was occupied with a detailed study of ecology, morphology and systematics and with the fitting together of the assembled data. It was only after this second phase was far advanced that the third, experimental, period began.

Judged against this background, bacteriology has had a highly atypical development. The natural historical period in this division of biology got under way only in the middle of the 19th century, far behind its sister sciences, and progressed slowly but surely for some fifteen years. Suddenly, largely through the experimental and intellectual genius of two men, Pasteur and Koch, the young science was catapulted into its third period and underwent an expansion during the last decades of the 19th century which, it is hardly too much to say, altered radically the conditions of physical existence in Western civilization.

<sup>1</sup> Present address: Division of Applied Biology, National Research Council, Ottawa, Canada

By 1900, the biotechnical applications of bacteriology had reached a level not yet attained in botany and zoology

One cannot deny the value of what has been accomplished in this way, but the unfortunate and inevitable consequence has been that most bacteriologists have centered their attention on thirty or forty important eubacterial parasites to the virtually complete neglect of the remaining thousands of species. Hence even today, our understanding of bacteria as *biological entities* remains fragmentary and disorganized. Morphology, taxonomy, ecology in the wider sense—none of these phases has even been surveyed, let alone developed in a systematic way. The consequent deficiencies in our fundamental knowledge are a continual source of misunderstanding and a bar to further scientific development whose seriousness is only now beginning to become generally realized. A striking example of the ill effects which have resulted from this one-sided expansion is provided by the work of the past twenty years on the cytophaga group.

These organisms were first recognized by Hutchinson and Clayton in 1919, and since that time have been studied by a considerable number of workers. The primary interest of the cytophagas has lain in their importance as one of the most active and widespread groups of cellulose-decomposers,<sup>2</sup> but the fascinating morphological, cultural, biochemical and taxonomic problems which they present have helped in no small measure to attract the attention of bacteriologists.

While much remains to be done, particularly from the biochemical standpoint, it can be said today with fair confidence that most of the outstanding problems connected with the cytophagas have been at least partially solved. In the main, these problems have really been extremely simple, much of the data necessary for their solution was already present in the classical paper of Hutchinson and Clayton. Yet such topics as the cycle of development, the systematic position and the carbon nutrition of these organisms have puzzled and confused able investigators and provided the material for more than one scientific controversy during the past twenty years. The reason for this slow progress can only be sought in our deficient basic knowledge, a handicap against which even the best bacteriologist must struggle in vain.

For the purposes of brief characterization, one may say that the cytophagas are primitive myxobacteria which differ from all other members of the class through their inability to form organized, discrete fruiting bodies. Certain species produce spherical unicellular reproductive structures known as *microcysts*, which are very similar in appearance and mode of formation to the microcysts of the higher genera *Myxococcus* and *Chondrococcus*, but which lie scattered at random among the vegetative cells instead of being grouped into organized

<sup>2</sup> The activities of the cytophaga group under natural conditions is a topic which deserves a chapter to itself. However, I have omitted all mention of this subject because an adequate treatment necessarily involves extensive discussion of the methodological principles underlying soil microbiological research, which is not germane to the present work. Readers interested in this phase should consult Waksman (1932, 1940), Winogradsky (1929, 1938), Jensen (1940) and Norman and Fuller (1942).

structures Other species never form microcysts, existing entirely in the vegetative state

The use in the following pages of the generic designation *Sporocytophaga*, recently proposed (Stanier, 1940) for the microcystogenous species, restricts Winogradsky's (1929) genus *Cytophaga*, which several students have used to cover the whole group, to the amicrocystogenous forms alone However, where descriptive terms such as "the cytophagas" or "the cytophaga group" are mentioned, it should be understood that they apply to all species, both microcystogenous and amicrocystogenous This usage, while not strictly correct, has been employed in the past and has the advantage of convenience

There are only two comprehensive publications on the cytophagas That of Winogradsky (1929) has been superseded by later work, while the more recent contribution of Imseneckı and Solntzeva (1936) is in Russian and thus largely unavailable to investigators outside the U S S R Consequently it has seemed desirable to combine the report of my own work with a review of the earlier, somewhat scattered and often contradictory literature A monographic treatment has been adopted, bringing together old and new information in a logical rather than a historical sequence

## METHODS

### *Enrichment cultures*

The cellulose-decomposing soil cytophagas can be enriched with a medium consisting of cellulose (usually in the form of filter paper) and a neutral or slightly alkaline mineral base containing either ammonium or nitrate salts as nitrogen source The exact composition of the mineral base seems to have little importance, and many variations have been proposed by different workers In my studies the following formula has proved satisfactory

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> or KNO <sub>3</sub>	0 1 g
K <sub>2</sub> HPO <sub>4</sub>	0 1 g
MgSO <sub>4</sub>	0 02 g
CaCl <sub>2</sub>	0 01 g
FeCl <sub>3</sub>	0 002 g
Tapwater	100 ml
pH adjusted to 7 0-7 5	

The enrichment cultures should be incubated under aerobic conditions at temperatures which can lie between 20 and 30 C Hutchinson and Clayton (1919) and others have used a liquid enrichment medium, but this is somewhat disadvantageous in that, owing to its accumulative nature, it tends to yield only one species, *Sporocytophaga myxococcoides* Markedly superior is the separative enrichment technique introduced by Winogradsky (1929), in which plates of silica gel, washed sand or agar, containing the mineral base, are covered with filter paper and inoculated at regularly spaced intervals with small particles of soil Usually only one or two types of cellulose-decomposer will develop around each soil particle, and thus a variety of forms can be obtained at once in a fair condition of purity The cytophagas appear on such plates after 4 to 5 days' incubation

tion at 30 C in the form of small yellow, orange or pink mucoid spots on the filter paper. In the course of a week these spots increase in size and often become translucent due to the complete dissolution of the fiber structure within them. This translucency is highly characteristic of cytophaga colonies, since there are no other cellulose-decomposing organisms which effect such a complete disintegration of the substrate in so short a time.

Winogradsky considered that silica gel possessed great advantages over agar for enrichment plates. I have used both, and cannot subscribe to this opinion. Agar, particularly if purified beforehand by washing with cold water for 10 to 14 days (a process which removes much of the extraneous organic matter) provides a base almost as selective as silica gel but without the inconveniences of preparation which encumber the use of the latter substrate. Most of the difficulties in the plate enrichment method are caused by overgrowths of filamentous fungi and actinomycetes, which can be reduced to a very low level by keeping the filter paper saturated with water. For this reason, plates with an agar concentration of 10 per cent are advantageous, since they dry out more slowly than ones with a higher percentage of agar.

There are indications (Johnson, 1932) that chitin-decomposing organisms belonging to the genera *Cytophaga* and *Sporocytophaga* can be isolated from terrestrial environments by the use of an enrichment medium with chitin as sole source of carbon and nitrogen, unfortunately, this work was of a preliminary nature and the subject has never been reinvestigated.

No suitable enrichment method is at present known for the marine cytophagas. Although occasionally identifiable morphologically in marine agar enrichment cultures, they are far outnumbered by the eubacterial agar-decomposers and do not appear on streaked plates.

### *Pure culture techniques*

Vigorous enrichment cultures of the soil cytophagas can be obtained in a state of fair purity, particularly by Winogradsky's method, but the purification of these, as of so many other cellulose-decomposing bacteria, has presented great difficulties which have only recently been overcome. Consequently, some workers (Winogradsky, Krzemieniewska, Walker and Warren) have contented themselves with the study of avowedly impure cultures, in fact Winogradsky, rejecting the bacteriological doctrines which have held sway since the time of Robert Koch, has even developed what one might term a philosophy of impure cultivation (1938). In the case of the cytophagas this procedure is satisfactory for morphological and general biological observations, since contaminating organisms (apart from other cellulose-decomposers) are kept at a very low level by the selective nature of the medium employed and can in any case be differentiated morphologically from the distinctive cytophaga cells. When it comes to studies on nutrition and metabolism, however, pure cultures are a *sine qua non*.

Hutchinson and Clayton (1919) claim to have obtained pure cultures of *Sporocytophaga myxococcoides* (= *Spirochaeta cytophaga* H and C) by the use of mineral agar plates containing finely divided cellulose. Since at first they regarded the microcysts of this organism as a contaminant, they spent much effort in attempts to separate it from the vegetative cells, the whole report of

their isolation procedure is confused by this, and it is not easy to tell at what stage the real contaminating forms were eliminated. The statement that butyric acid was among the products of cellulose decomposition by *S. myxococcoides* makes it doubtful, as pointed out by Winogradsky (1929) and Imsenecki and Solntzeva (1936), whether their cultures ever were actually pure. Since acidic products have never been found by other workers as a result of cellulose decomposition by cytophagas it is not impossible that Hutchinson and Clayton's cultures were contaminated by butyric clostridia.

Bokor (1930) also employed mineral agar plates containing finely-divided cellulose, on which he claimed to have obtained pure cultures of *S. myxococcoides*. However, his strain was clearly contaminated with an actinomycete, which he took for a stage in the life cycle of the cytophaga.

Stapp and Bortels (1934), again employing poured cellulose agar plates, were the first to isolate indubitably pure cultures of *S. myxococcoides*. Their success where others had failed was probably due to the use of a low agar concentration which, as shown later by Jensen (1940), is a factor of cardinal importance. However, the same technique proved unsatisfactory for the isolation of amicrocystogenous cytophagas. This is difficult to understand in view of the successful outcome of similar attempts by Jensen and myself. Single-cell isolation, the "negative" method of Winogradsky, and the dilution method gave no better results. Stapp and Bortels finally reached the conclusion "dass die Gewinnung von Reinkulturen bei diesen *Cytophaga*-Arten einfach nicht möglich ist", and fell back on the hypothesis that the amicrocystogenous forms lived "symbiotically" with non-cellulose decomposers, an hypothesis since exploded by Jensen. This "symbiotic" effect has also been invoked by workers with the anaerobic cellulose-decomposers as a last resort to explain failure in obtaining pure cultures. Such claims should, in my opinion, always be taken with a grain of salt. In the ultimate analysis a symbiotic effect is explicable on either physical or chemical grounds or a combination of both so that with sufficient persistence and ingenuity a separation of the two forms should be possible. In any case, the body of evidence adduced does not yet bring conviction that a true example of mutualistic symbiosis exists among the cellulose-decomposing bacteria.

A new method for the purification of microcystogenous cytophagas was developed by Imsenecki and Solntzeva (1936). It takes advantage of the fact that microcysts show a slightly greater thermal resistance than the non-spore-forming eubacterial contaminants, so that by heating of microcyst suspensions, repeated if need be, the latter can be eliminated. The authors reported lack of success with other methods.

Jensen finally evolved a completely satisfactory method, using poured plates of cellulose-agar, by means of which he readily obtained pure cultures of a number of strains of *Sporocytophaga* and *Cytophaga*. He found that the critical factors, presumably neglected by earlier workers, were a low agar concentration and a very finely divided cellulose suspension. The latter was prepared according to the method of Scales as modified by Kalnins (1930).

Following Jensen's directions, I have had no difficulty in purifying several

strains of *S. myxococcoides*, *C. hutchinsonii* and *C. rubra*. The reasons for the success of this technique are not hard to discover. The cytophagae have to be in direct contact with cellulose in order to attack it, so that unless the cellulose is very finely divided and evenly dispersed throughout the medium it will be largely unavailable to them. The use of a *weak* agar gel allows creeping movement to take place, so that the organisms, instead of being confined to a small area where the food supply is rapidly used up, can creep outwards in all directions through the agar. Contaminating flagellated bacteria, on the other hand, cannot move through a 10 per cent agar gel (unless possessing the ability to liquefy agar) and are thus immobilized and localized. This interpretation is borne out by the appearance of the growth: cytophagae never occur as macroscopically visible colonies surrounded by areas of cellulose dissolution, such as one finds with other cellulose decomposers. Instead, spreading, translucent, yellow or pink "windows" are formed, within which there is an even, thin distribution of cells. There is never any evidence of enzymatic zones around these "windows"; attack on the cellulose takes place only in the immediate neighborhood of the organisms. In view of the discovery (Stanier, 1942c) that the cytophagae can be grown with glucose sterilized by filtration as a carbon source, the purification procedures can now be simplified even further by streaking either directly from enrichment cultures or from the first poured cellulose plates onto filtered glucose agar plates.

For the maintenance of stock cultures, tubes of liquid media with strips of filter paper partly immersed have been universally used. If kept at room temperature such cultures will remain viable for at least three months.

The purification of the marine cytophagae offers no difficulties, since they grow well and spread rapidly on sea-water peptone agar. This medium, when buffered with  $\text{CaCO}_3$  to counteract the acidity resulting from agar decomposition, is also the best for stock cultures. In the absence of  $\text{CaCO}_3$  cultures die after a week or two.

#### *Methods of morphological examination*

The extremely low refractivity of vegetative cytophagae cells, coupled with their growth in and on a solid substrate, makes the observation of living preparations in the light field very difficult. This was partially remedied by the use of cellophane as a substrate (Krzemiemska, 1933, Stapp and Bortels, 1934). Stapp and Bortels also resorted to dark-field illumination. Since the discovery of growth on glucose the problem has been much simplified, and I have made almost all my observations on living cells of the soil cytophagae from glucose cultures. The above difficulties do not apply to the marine cytophagae with their less restricted nutrient requirements and greater size.

A second problem where morphological studies are concerned has been that of finding suitable staining methods. The majority of conventional bacteriological stains are entirely unsatisfactory. Hutchinson and Clayton obtained fair results with hot carbol-fuchsin, but this stain leaves masses of color on the cellulose fibers and the slide. An excellent method, evolved by Winogradsky (1929), is the use of phenol-erythrosin followed by dilute aqueous gentian violet, which

stains cells very intensely while leaving cellulose almost colorless. Krzemieniewska (1930) introduced the use of Giemsa's stain, sometimes followed by tannin differentiation. The methods of both Winogradsky and Krzemieniewska can be used to demonstrate the behavior of chromatin material during microcyst formation, giving similar cytological pictures. I have found Winogradsky's stain to be preferable.

The method of fixation is also important. Heating of wet smears, as is customarily done by bacteriologists, will result in gross distortions like the "spirochaetal" artefacts. Winogradsky allowed his preparations to dry and then fixed them by immersion in 95 per cent ethyl alcohol, "que l'on brûle en soufflant plusieurs fois dessus au moment même où il s'allume". Even better, in my experience, is fixation of the moist preparation by exposure to osmic acid vapors. However, as will be discussed later, none of these methods successfully preserves the shape of the living cell. Fixation and staining should be used only if one wishes to observe the changes in chromatin material accompanying microcyst formation or the arrangement of the cells on attacked fibers, for the rest, it is safer to depend on the examination of living cells.

It is almost impossible to obtain good stained preparations of the marine agar-decomposing cytophagas. If smears are made with tap or distilled water the organisms become distorted, while smears with 3 per cent NaCl or seawater undergo equally undesirable plasmolytic changes on drying. Of all the methods tried, negative staining with a saturated aqueous nigrosin solution gave the best results.

#### MORPHOLOGY AND LOCOMOTION

##### *The vegetative cells*

The vegetative cells of the cytophagas are slender, gram negative rods characterized by an extreme flexibility, which is far greater than that of most other myxobacteria. Another outstanding feature, particularly of the soil cytophagas, is the already noted low refractility, which makes living cells hard to see. This has been attributed by Winogradsky (1929) to their small size, however, stained preparations show that their dimensions are not less than those of many easily visible true bacteria. The low refractility is perhaps caused by the absence of a cell wall.

The opinion expressed by Winogradsky and most subsequent investigators that normal cytophaga cells are spindle-shaped and pointed is incorrect. When examined *in the living state* none of the species which I have studied conform to this description. Living cells of soil and marine cytophagas alike are only very slightly (if at all) spindle-shaped, and have rounded ends for the most part. Wet mounts prepared from cultures grown under many different conditions, irrespective of whether they are observed with light- or dark-field illumination, never fit the classical description. It is only in fixed and stained preparations that the fusiform cell shape becomes apparent, in such preparations virtually every cell is spindle-shaped and pointed. This indicates the danger of assuming that techniques devised for the study of eubacterial forms can be used equally well for other bacterial groups. True bacteria, because of their rigid cell walls,

may be studied morphologically from stained mounts without much chance of error, the fragile cells of the cytophagas (and also of other myxobacteria) become grossly distorted when fixed and stained in the customary bacteriological manner

Healthy, living cells are usually straight or slightly curved, although marked deformations occur during locomotion and flexing movements. The spirally twisted filaments described and figured by Hutchinson and Clayton (1919) for *S. myxococcoides* are artefacts, as I have suggested before (Stanier, 1941). Among the soil cytophagas they can never be found among living cells and are extremely rare in carefully prepared stained mounts, particularly if osmic acid fixation of the wet preparations be used and heating avoided. Similar artefacts are conspicuous in nigrosin preparations of *C. krzemieniewskae*.

Division is invariably by constriction. The two daughter cells gradually draw apart, while the connecting cytoplasm becomes thinner. Among the cytophagas this characteristically myxobacterial mode of division can be seen most clearly in the large cells of *C. krzemieniewskae*.

In old cultures of *C. diffluens* long, threadlike involution forms are common (Stanier, 1940). They can also be found in cultures of the soil cytophagas, especially when peptone or yeast extract is supplied as nitrogen source, and in old cultures on glucose. Another type of involution form common in the amicrocystogenous soil cytophagas (Krzemieniewska, 1933) and in *C. krzemieniewskae* consists of round, swollen bodies superficially resembling microcysts, but only faintly visible in the unstained condition and with weak staining properties.

### *Microcyst formation*

Several of the early workers on aerobic cellulose decomposition—van Iterson (1904), Merker (1912), von Gescher (1922), Löhnis and Lochhead (1923)—observed in their enrichment cultures a large coccoid form, which often entirely covered the fibers as a "Mikrokokkenschleim". This organism was not obtained in pure culture, and opinion as to its actual role in the decomposition of cellulose varied. Van Iterson considered it to be a non-cellulose-decomposing micrococcus whose presence favored the activity of a highly motile, non-sporeforming rod, *Bacillus ferrugineus*, which he held to be the active agent of cellulose breakdown. Merker, on the other hand, regarded the coccus as the cause of cellulose decomposition in his cultures, in token of this he gave it the name *Micrococcus cytophagus*.

These findings were clarified by the work of Hutchinson and Clayton (1919). In common with the earlier investigators, Hutchinson and Clayton found a large coccus-like form in many of their enrichment cultures, occurring along with thin, flexible rods (the vegetative cytophaga cells) which, presumably because of their low refractivity, had been overlooked by van Iterson and Merker. During the course of purification these two forms persisted together, usually in young cultures the rods predominated, but in the course of time the cocci appeared and were ultimately present in large numbers. All attempts to separate them were fruitless. The crucial experiment, which finally convinced Hutchinson and Clayton that rod and coccus belonged to the life cycle of a single organism,

consisted of the preparation of two consecutive sets of dilution cultures. In one of the highest dilutions of the first set an apparently pure growth of the rod form was obtained. A second series was immediately prepared from this, but after four days' growth even the highest dilutions of the second set contained a mixture of rod and coccus. On the assumption that these two forms were separate entities, this would have implied a concentration of 40,000,000 coccus forms per milliliter of inoculum, such a large number could not possibly have been present, however, without being observable.

Once convinced of the causal relationship existing between these two forms, Hutchinson and Clayton began a search for intermediate stages. They observed a series of forms which, starting from the thin sinuous rods, became progressively shorter and thicker. At the same time, staining showed that the originally evenly distributed chromatin material had undergone a condensation, finally taking up a central position in the cell in the form of a transverse band. Following this stage it was claimed that transverse fission occurred, the two resulting oval cells (each with a round excentric chromatin body) gradually changing into the coccoid form, which in the mature state stained evenly and intensely. Return to the rod form was figured as occurring by release of the typical long thin vegetative cell from the interior of the coccus. The designation of "sporoid" was given to the coccoid form, since the authors felt that "the use of the term 'cyst' might imply more than is perhaps warrantable at the present time."

Here the matter remained until 1929, when Winogradsky published the results of his extensive studies on aerobic cellulose decomposition. By the use of his superior separative enrichment technique he isolated a series of species whose vegetative morphology was similar to that of *Spirochaeta cytophaga*, but none of which formed sporoids. One of these appeared to be identical with Hutchinson and Clayton's organism in all other respects. Winogradsky apparently saw large coccoid bodies in some of the cytophaga colonies on enrichment plates, but regarding them as contaminants he passed such colonies by in favor of others, considered similar, which showed only the rod form. Winogradsky recognized that his organisms differed sharply from the true spirochaetes, and proposed the new generic name *Cytophaga* for the group. The type species was given as *C. hutchinsoni*, the non-sporoidforming organism which Winogradsky regarded as identical with *Spirochaeta cytophaga*.

The conflicting results of Hutchinson and Clayton and of Winogradsky were soon clarified by Krzemieniewska (1930, 1933), whose careful and exact morphological studies established the existence of two species of *Cytophaga*, superficially very similar but differing in their cycles of development. In its main outlines, she confirmed the work of Hutchinson and Clayton with respect to the developmental cycle of *Spirochaeta cytophaga*, establishing at the same time the absence of a sporoid stage in Winogradsky's *Cytophaga hutchinsoni*. She was able to follow in detail the germination of the sporoids and their transformation into vegetative cells, thus decisively refuting Winogradsky's contention that these bodies were ordinary cocci present as contaminants. Like the English authors, she noted the shortening and rounding up of the vegetative cells and the concomitant changes in the distribution of chromatin material, which she examined

with great care (For cytological details, see Krzemieniewska, 1930) However, she was unable to confirm the claim that transverse fission occurred at one stage of sporoid formation This claim was probably based on the observation of two contiguous cells at the same stage of development with a polar distribution of chromatin in the neighboring ends, I have seen such an arrangement on several occasions, and it resembles closely Hutchinson and Clayton's illustrations of "dividing" intermediate forms

The process of germination as observed by Krzemieniewska differed from that suggested by Hutchinson and Clayton The sporoids, surrounded by a mucoid sheath, first showed a renewed segregation of the chromatin material into discrete intracellular bodies, following this, the mucoid sheath disappeared from one side and the enclosed cell emerged and underwent a slow elongation to become once more a vegetative cell During cell elongation the chromatin went through a series of changes of unknown significance, which ultimately resulted in an even dispersal throughout the cell

Krzemieniewska recognized the similarity of this developmental cycle to that which occurs in myxobacteria belonging to the family *Myxococcaceae*, and for this reason she renamed Hutchinson and Clayton's organism *Cytophaga myxococcoides* To the sporoid she gave the more accurate designation of *microcyst*

At first Winogradsky (1932) discounted Krzemieniewska's findings, but ultimately he accepted them (1935) Since then the developmental cycle thus established has been confirmed by a number of workers, although there have been differences of opinion as to the exact mechanism of microcyst germination Stapp and Bortels (1934) have described the outgrowth of the vegetative cell from the main body of the microcyst, which gradually decreases in size and is ultimately thrown off as a minute spherical body Much the same type of germination has been described by Imseneck and Solntzeva (1936) both for *S. myxococcoides* and for *S. ellipsospora*, a new species discovered by them Issatchenko and Wakengut (1932), on the other hand, claim to have seen the vegetative cell lying in the form of a ring within the microcyst, from which it is released later My own studies on microcyst germination in *S. myxococcoides*, based entirely on the examination of wet mounts, support Krzemieniewska's account of the process In such wet mounts the castoff sheaths formerly enclosing microcysts look somewhat like mature microcysts containing ring-shaped vegetative cells, at least on superficial examination The conclusions of Issatchenko and Wakengut may have been based on the misinterpretation of these bodies

Stapp and Bortels were the first to observe the interesting phenomenon of star formation ("Sternbildung") In many liquid cultures there occur round masses of close-packed cells sometimes as much as 30-40  $\mu$  in diameter On careful examination the cells can be seen to radiate out in all directions from the center of the mass The structure bears a curious superficial resemblance to a chalk sphaerolite, this is particularly well shown by the dark-field photomicrograph in Stapp and Bortels' paper These authors occasionally found transitional forms between vegetative cells and microcysts within stars from cultures of *S. myxo-*

*coccoides*, and drew the conclusion that star formation was in some way connected with microcyst formation. This is a tempting hypothesis, since it implies that the stars might be homologous with the organized fruiting bodies of the higher myxobacteria, however, I do not believe that it is valid. I have examined a large number of stars from cultures of *S. myxococcoides*, and although they occasionally contain transitional forms, the number is not greater than among the surrounding unorganized cells. Furthermore, star formation is extremely marked in cultures of the marine cytophagae, which never form microcysts. It is probably a physical phenomenon, perhaps connected with slime secretion by cells growing in a liquid medium.

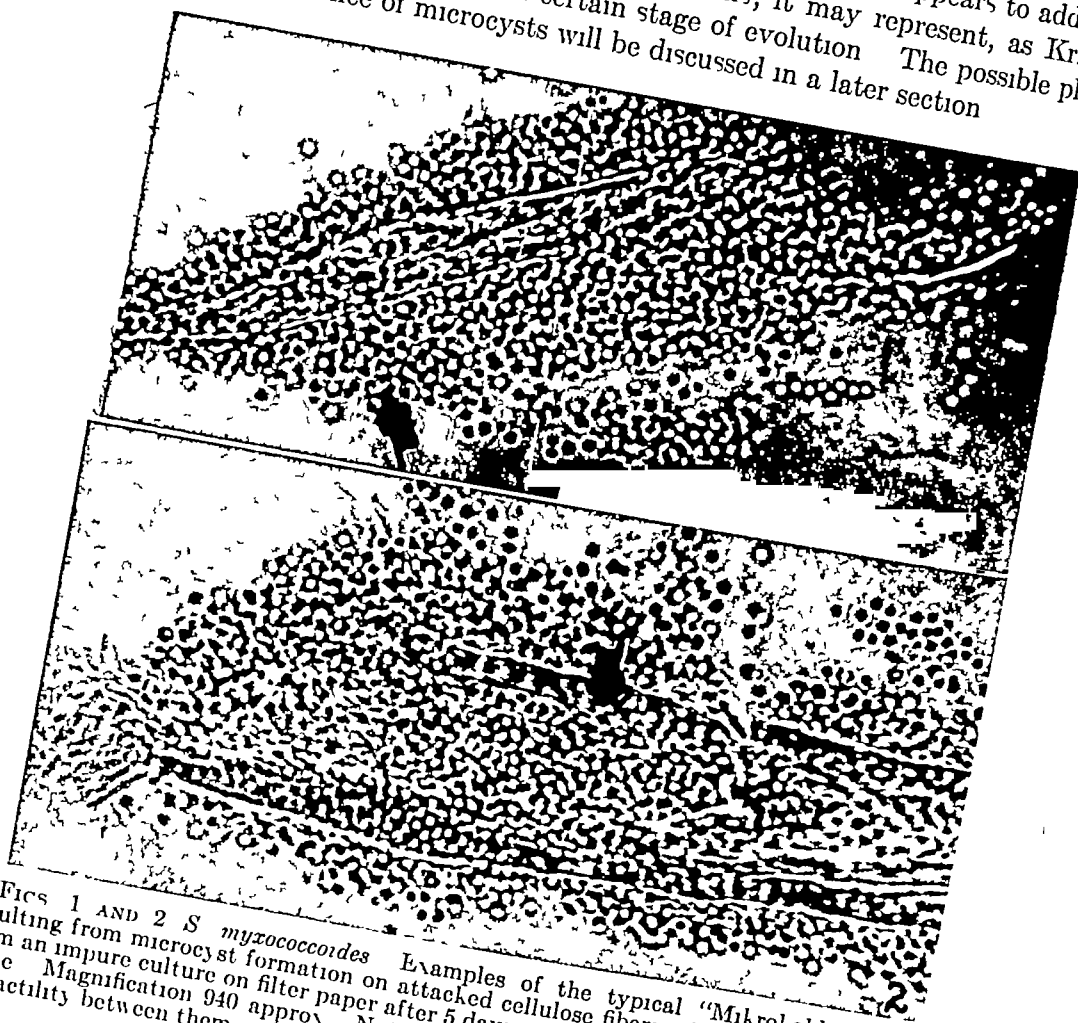
What, in general, are the conditions for microcyst formation? Imseneck and Solntzeva found that the process was suppressed under conditions of low oxygen tension, a fact which I can fully confirm. Only in those portions of cultures freely exposed to the air does widespread microcyst formation occur. A second important factor, not apparently previously recognized, is temperature. Microcysts are formed most abundantly at 30 to 35 C, while at 20 C the process may be entirely suppressed. Strains maintained continuously at room temperature may not form microcysts for months at a time, but when placed at a higher temperature the process becomes established again.

Imseneck and Solntzeva have suggested the possibility of obtaining amicrocystogenous races, although they were not actually successful in so doing, by keeping cultures continuously at a low oxygen tension. However, the gradual diminution of microcyst formation in pure cultures, even under optimum conditions, is a very marked phenomenon. Only in still impure or recently purified strains of *S. myxococcoides* can one observe the typical picture of a "Mikrokokkenschleim" around the cellulose fibers, as illustrated in figs 1 and 2. Nevertheless, with one possible exception none of the strains of *Sporocytophaga* which I have studied has lost completely the ability to form microcysts. The possible exception was a strain originally very similar to *S. ellipsospora* isolated by Dr H. L. Jensen and sent to me with the comment that it appeared to have become non-microcystforming. I was likewise unable to find microcysts, but unfortunately the organism died off before rigorous tests could be made. From the taxonomic aspect this is an important problem, which should be carefully investigated in the future.

The resistance of microcysts to adverse conditions is very slight. Krzemieniewska (1933), who first investigated this, reported that the thermal death point of the microcysts in *S. myxococcoides* was 62 C, while that of the vegetative cells was 58 C. Imseneck and Solntzeva (1936) have given the somewhat higher figure of 68 C for the microcysts in this species, but they found development from suspensions heated to 64 C and 66 C to be erratic. The thermal death point for microcysts of *S. ellipsospora* is 58 C (Imseneck and Solntzeva), no figures for the vegetative cells of this species are available.

Cultures of *Sporocytophaga* are remarkably resistant to drying, Imseneck and Solntzeva report development after 60 days' desiccation, Krzemieniewska after six months. However, the preparations used contained vegetative cells, and hence the role played by microcysts is not certain. Krzemieniewska found that the vegetative cells could survive 16 days' drying, but she did not continue the experiment over a longer period.

Microcyst formation, on the basis of present knowledge, appears to add little to the powers of resistance of these organisms, it may represent, as Krzemińska has suggested, only a certain stage of evolution. The possible phylogenetic significance of microcysts will be discussed in a later section.



FIGS 1 AND 2 *S. myzococcoides*. Examples of the typical "Mikrokokkenschleim" resulting from microcyst formation on attacked cellulose fibers. The material was taken from an impure culture on filter paper after 5 days at 30 C, and photographed in the living state. Magnification 940 approx. Note the vegetative cells in fig 2 the difference in refractility between them and the microcysts is clearly apparent.

### Motility

For a long time the method of locomotion of the soil cytophagous was obscure, although the way in which they grew on cellulose fibers implied that some movement must occur. Hutchinson and Clayton (1919) observed rotatory movements and displacements in hanging drops, but Winogradsky (1929) could not confirm this, and suggested that since movement occurred on a solid substrate, it was probably of a climbing or creeping type. Neither of these workers could demonstrate flagella but Winogradsky, seeking evidence for locomotor structures, believed that he had seen extremely small "haustoria," which were on the borders of visibility, emerging from the cell wall. This has not been confirmed.

by later workers Stapp and Bortels (1934) and Imseneckı and Solntzeva (1936) finally showed that locomotion among the soil cytophagas was of the creeping type characteristic of myxobacteria. This was subsequently confirmed in the marine forms (Stanier, 1940).

The failure of Hutchinson and Clayton and of Winogradsky to observe definite movement is now understandable, myxobacteria are always completely immotile when suspended in a liquid. For motility to occur, it is necessary for the cells to be in contact with a *surface*, either solid or liquid—a water-film, glass, agar, cellulose, or even other cells.

The simplest way to study movement of the individual cells is to make a wet mount and focus on the underside of the coverslip, to which numerous cells always adhere. Many of these are attached only by one tip, and undergo the peculiar “pendelnde Bewegungen” first described in myxococci by Baur (1905), they swing rapidly back and forth in the liquid, sometimes hitting the glass with the entire body, in which case they may adhere to it. Others can be seen gliding evenly along the glass surface, the ends of a moving cell may vibrate markedly, and the even, progressive motion is often interrupted by flexing movements. A cell can, and often does, stop suddenly and start travelling in the reverse direction. Flexing of the cells also occurs independently of linear movement. What the reason for such flexing movements in otherwise stationary cells may be, it is impossible to say, at any rate, they are not symptoms of degeneration as Stapp and Bortels believed. I have examined the process in the soil cytophagas as well as in the marine forms, and can fully confirm my previous contention (1940) that it is always of very marked occurrence in the majority of young, healthy cells which are lying in contact with a surface. On the other hand, the absence of any flexing movements is often a sign of senescence.

The statement (Stanier, 1940) that the unit of effective movement in the marine cytophagas appeared to consist of 20 to 30 cells is true only during the extension of a swarm (*vide infra*), in wet mounts these organisms, like the soil cytophagas, can creep actively as individuals.

Linear locomotion, as well as flexing and swinging movements, is markedly affected by oxygen tension and temperature. In crowded wet mounts, all movement ceases after a short time except near the edge of the coverslip and in the neighborhood of air bubbles. The influence of temperature on the rate of movement has already been noted by Stapp and Bortels, and Imseneckı and Solntzeva. Movement of the soil cytophagas is very noticeable at 28 to 30 C, as the temperature falls it slows down, and at 20 C is only very slight. The phenomenon is worthy of further study, it may throw some light on the mechanism of creeping movement, which has long been and still remains a major unsolved problem in biology.

The maximum observed rate of movement in wet mounts of the soil cytophagas (temperature uncontrolled) is approximately 150  $\mu$  per minute. That of the marine species is much less, not more than 30 to 50  $\mu$  per minute.

### *Swarming*

In almost all members of the *Eubacteriae*, the macroscopically visible masses of cells which we term "colonies" extend their dimensions only passively, as a result of the action of mechanical forces. The young cells at the edge of a colony are sometimes pushed outwards by the cell accumulations behind, in other cases, particularly in such groups as the actinomycetes and aerobic sporeformers where there is a mycelial or filamentous vegetative structure, size increase may be due to a direct outgrowth from the colony's periphery. Among the thousands of species in the *Eubacteriae*, there is only a handful of exceptional forms, the outstanding example being the *Proteus* group, in which colony extension occurs actively.

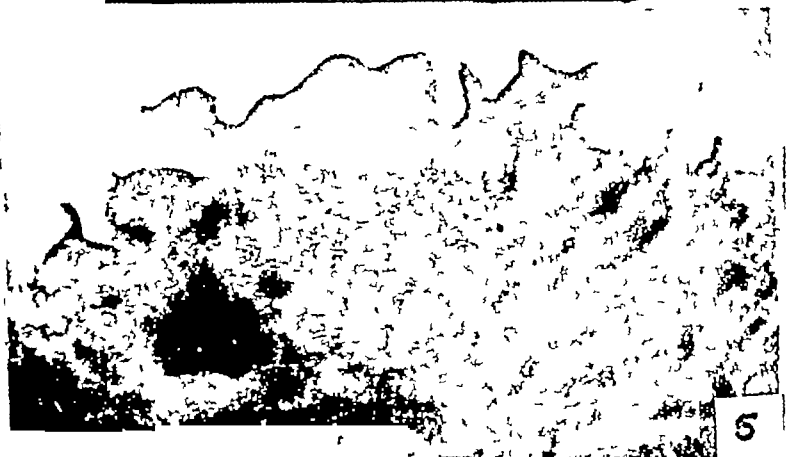
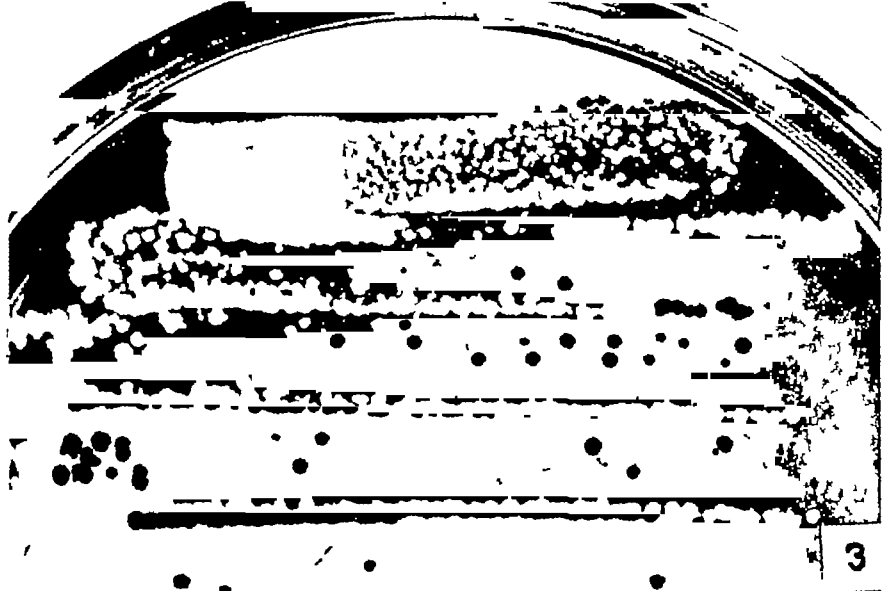
On the other hand, increase of colony size in the *Myxobacteriae* as a whole, cytophagas included, is characteristically an *active process*, an expression on a large scale of the creeping locomotion of the individual cells. It occurs by continuous extension from the periphery of long, often pointed, cell masses, which creep out across the substrate like miniature armies. For this reason Jahn (1924) gave the name "swarm" to the myxobacterial colony.<sup>3</sup>

The process of active swarm extension or swarming reaches its peak among the *Polyangiaceae*, in certain members of this group the large majority of the cells composing a swarm are regularly located in the advancing periphery, while the older, central portion of the swarm contains only a few isolated vegetative cells which have been left behind (Jahn, 1924). Among the myxococci and the cytophagas, however, the center of the swarm always contains many organisms, and the extending periphery is a flat, almost invisible region only a few cells thick.

Swarming can be observed even under the low-power lens of the microscope, but for detailed study coverslip preparations are best used. The marine cytophagas provide excellent material for this purpose, since their cells are large and the mass movement is extremely rapid (see Stanier, 1940, for an account of the process).

Until recently similar studies on the soil cytophagas were not possible because these organisms could not be grown on the surface of a clear agar medium. Since the discovery of growth on glucose, I have examined the process of swarming on glucose-agar plate cultures of *S. myxococcoides*, *C. hutchinsonii* and *C. rubra*. Only in the former species is swarming marked and typical. At first it occurs only in some of the colonies, a curious case of colony dimorphism. On plates less than a week old, many of the colonies are very small and hemispherical, with sharply defined edges. In colonies of this type swarming does not develop until considerably later. In other colonies which, although somewhat raised in the center, have a flat periphery, swarming masses extend out from all sides at a very early stage.

<sup>3</sup> The descriptive term "pseudoplasmodium" used by Thaxter (1892) in reference to the myxobacterial colony, is an undesirable one since it suggests a connection with the myxomycetes, and more particularly with the *Acrasidae*, to which myxobacteria are not even remotely related.



- FIG 3 *C. hutchinsonii* Plate culture on 10 per cent glucose agar after one week at 30°C, showing the compact, cubacterium-like nature of the colonies
- FIG 4 *C. hutchinsonii* Edge of a young colony on glucose agar, showing swarming × 13
- FIG 5 *S. myzococcoides* Edge of a swarm on a 0.1 per cent glucose agar plate × 13

In *C. hutchinsonii* swarming is largely suppressed and the colonies are superficially indistinguishable from those of a true bacterium. However, on young vigorous plate cultures a few typical moving masses can be found extending out for a short distance from some of the colonies. It is probably the copious synthesis of a microbial gum in this species which prevents normal swarming.

In *C. rubra* swarming is never apparent. The colonies are always very small and inconspicuous, attaining a diameter of 2 mm at the most. They have a hazily defined and indefinite periphery. Instead of extending out across the surface, the cells creep downward into the agar, so that when scraped with a platinum needle only a small part of the colony can be removed.

*The effect of macromolecular orientation in the substrate on movement  
and arrangement of the vegetative cells*

A striking characteristic of the soil cytophagous is the pattern of cell arrangement on attacked cellulose fibers. The extreme regularity of the investment has been remarked on by several workers, Winogradsky's (1929) comments in this connection are particularly cogent.

"Ce qui attire l'attention déjà dans les préparations faites avec un flocon arraché d'une tache jaune à peine naissante, c'est la densité et la régularité de la disposition de ces cellules sur les parois des fibres attaquées: ils y forment un revêtement régulier composé d'individus logés parallèlement et orientés dans la même direction. On y observe souvent des tableaux frappants qui paraissent démontrer qu'ils s'y fixent en s'adaptant en quelque sorte à la structure de la membrane qu'ils garnissent. Ainsi, s'ils habillent une fibre, dont les parois ont une structure spiralée, on les voit dirigés dans la direction de la spirale, par exemple de gauche à droite sur la paroi supérieure de la fibre couchée devant l'observateur, mais de droite à gauche, en suivant toujours le tour de la spirale, du côté opposé."

This is difficult to illustrate in photomicrographs, some disturbance of the orderly structure always occurs in stained preparations, and the plane of focus under oil immersion is too narrow to compass the entire width of a fiber. However, figs 6 to 12 give some idea of the appearance.

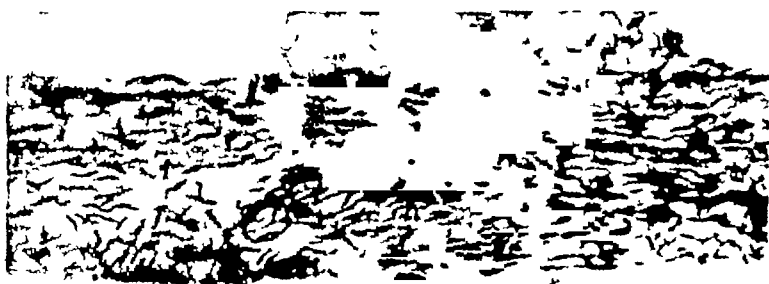
One should add to Winogradsky's lucid description only that the orderly arrangement becomes lost on those portions of the fibers which have reached an advanced stage of decomposition, when the fiber structure is largely dissolved, the cells have for the most part a random orientation.

Since studies on cotton fibers (e.g., Anderson and Kerr, 1938) have shown that the cellulose micelles are laid down in spirals around the fiber, Winogradsky's suggestion of an adaptation of the cells to the underlying structure of the substrate is no fanciful flight of the imagination. The demonstration (Stanier, 1942b) that among higher myxobacteria the orientation of the vegetative cells and the direction of their movement can be controlled by stresses in the agar serving as substrate provides further support for this view. In fact, there can be little doubt that the orientation of the cytophagous on cellulose fibers is another example of the conditioning of movement on solid surfaces by "ultrastructural"

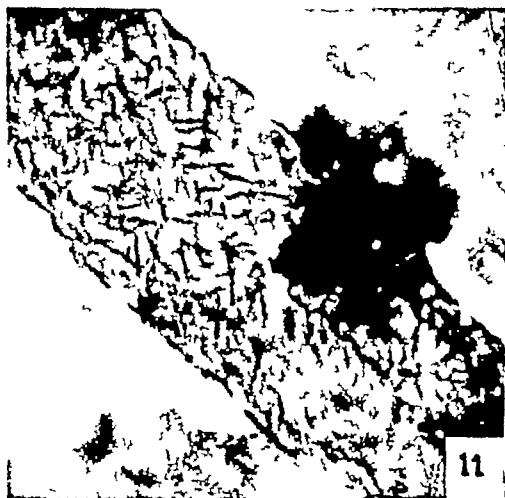


Figs 6-9 *S. myzococcoides* Attached filter paper fibers, showing both microcysts and vegetative cells Winogradsky's stain  $\times 920$

organization of the substrate, a phenomenon which, following the nomenclature of Jacobsen (1907), I have termed an *elasticotaxis* (Stanier, 1942b)



10



11



12

FIGS 10 AND 11 *C. hutchinsonii* Attacked filter paper fibers Winogradsky's stain  
 × 835  
 FIG 12 *C. rubra* Attacked filter paper fiber Winogradsky's stain × 835

On gelatin and agar, elasticotactic and elasticotropistic behavior is evident only after a micellar orientation of the substrate has been induced through the

application of mechanical tension A cellulose fiber, on the other hand, possesses a high degree of intrinsic orientation, and hence the application of mechanical tension is not necessary to produce elasticotactic responses

#### PHYSIOLOGY OF THE SOIL CYTOPHAGAS

##### *Carbon nutrition*

As has already been made clear, the soil cytophagas are a group highly specialized for the aerobic breakdown of cellulose In order to appreciate what this means from the standpoint of their metabolism, we must first consider briefly the physical and chemical nature of cellulose

The cellulose molecule is a highly polymerized, long-chain polysaccharide made up of  $\beta$ -glucose units linked together in the 1 4 position The lower size limits for the molecule are thought to lie between 100 and 200 glucose units, but the methods which have been used for molecular weight determinations are not entirely reliable, and the actual molecular size may well be much greater It is not improbable that the material which we call cellulose in reality consists of a whole range of substances of high, but varying molecular weight, with the same basic structure and similar physical and chemical properties The orthodox chemical concepts of molecular weight and molecular size lose much of their validity when one attempts to apply them to highly polymerized natural organic materials Physically, natural cellulose is a fibrous substance completely insoluble in water and organic solvents

The macromolecular nature and insolubility of cellulose have two obvious consequences from the standpoint of its availability as a food material for micro-organisms

- 1 Cellulose as such cannot enter the microbial cell

- 2 Therefore, the large, insoluble cellulose molecules must be decomposed in some way externally to the cell so as to yield soluble breakdown products which can enter the cell and be used for synthetic purposes, for energetic purposes, or for both

These deductions do not rest solely on theoretical considerations, they have been repeatedly shown to hold true in other cases of polysaccharide breakdown by a wide variety of living organisms

What are the simpler soluble organic compounds which might be released and used as a result of cellulose breakdown? One would expect the decomposition of cellulose to proceed by hydrolysis or phosphorolysis with the ultimate production of glucose Biochemical experience has shown beyond any doubt that in the overwhelming majority of cases polysaccharide decomposition eventually results in the release of the constituent simple sugars, which are then further broken down intracellularly by an oxidative or fermentative mechanism

Although both logic and general experience might have led one to anticipate some such mechanism for the breakdown of cellulose by the soil cytophagas, the findings of a whole series of workers investigating these organisms have been at best inconclusive, at worst directly opposed to the scheme outlined above It is not too much to say that the manner of cellulose decomposition and the carbon nutrition of the soil cytophagas have been major mysteries of microbial biochemistry during the past twenty years

These problems were first extensively explored and clearly stated by Hutchinson and Clayton in 1919. Their conclusions were as follows:

"*S. cytophaga* (= *S. myxococcoides*) appears to be specific in its relation to carbon sources and its requirements are only met by cellulose. Not only are other compounds (higher alcohols, organic acids and various carbohydrates) unsuitable for nutrition of the organism, but some, namely compounds possessing marked reducing properties, have been found to be toxic even in very low concentrations, e.g., 0.018 per cent maltose or 0.050 per cent dextrose."

Their experiments showed that *S. myxococcoides* was even more sensitive towards glucose and other soluble organic compounds than the chemosynthetic nitrifying bacteria, which had previously been regarded as the supreme examples of organisms adversely affected by "bons aliments". However, even though no good explanation of the toxic action of organic compounds on chemosynthetic bacteria has ever been offered, the phenomenon could be dismissed with the remark that these organisms were, after all, inorganic. *S. myxococcoides*, on the other hand, exists in nature solely through its ability to break down one of the most resistant and chemically one of the most complex organic compounds known, so that its peculiar behavior in this respect, equally inexplicable, was far more unexpected.

Although, as mentioned previously, Winogradsky (1929) never obtained pure cultures of the amicrocystogenous soil cytophagae with which he worked, and hence could not undertake a detailed examination of the problem of their carbon nutrition, his observations pointed very clearly to a like specificity of carbon requirements in these species. With the publication of Winogradsky's researches, the belief became firmly established that the whole cytophaga group consisted of obligate cellulose-decomposing bacteria.

This conclusion was further strengthened by the studies of Krzemieniewska (1930, 1933), Stapp and Bortels (1934), Imseneck and Solntzeva (1936), Bucksteeg (1936), Walker and Warren (1938) and Jensen (1940). None of these investigators ever obtained growth of any soil cytophaga in the absence of cellulose.

Stapp and Bortels (1934) filled an important gap among the carbohydrates, which Hutchinson and Clayton had tested for their possible effects on the cytophagae, by showing that the simple polysaccharides derived from the breakdown of cellulose (cellotetrose, cellotriose and cellobiose) were unutilizable and almost as toxic as glucose.

Hutchinson and Clayton, in discussing their results, had drawn attention to the singular fact that reducing compounds were particularly toxic. For example, 0.1 per cent glucose completely inhibited growth and cellulose decomposition by *S. myxococcoides* in their experiments, whereas 1.25 per cent sucrose only delayed the onset of development, and complete inhibition with this compound was not attained until 2.5 per cent was used. They suggested that the toxicity of reducing sugars might be caused by interference with the aerobic system of respiration of the organism, and pointed out that the opposite effect, a stimulation, often resulted from the addition of such reducing substances as glucose to cultures of strict anaerobes. Imseneck (1941) took up the problem anew from this standpoint, and was able to show that the toxic effect of glucose was quite unconnected with its

reducing properties His strain of *S myxococcoides* was completely inhibited by 0.3 per cent glucose, but could grow well at an oxidation-reduction potential much lower than that which resulted from the addition of this concentration of glucose to a mineral cellulose medium He also made the interesting observation that the "toxic" effect was not lethal but merely growth-inhibiting

Such was the status of the problem at the time when my investigations were begun The discovery of marine cytophagas with much less restricted nutrient requirements (Stanier, 1940) encouraged the belief that the carbon nutrition of the soil forms might be broader than had been previously expected However, a repetition of much of the previous work, for which *S myxococcoides*, *C hutchinsonii* and *C rubra* were used, yielded results in substantial agreement with those of the earlier investigators In no case was growth obtained on a medium devoid of cellulose I was able fully to confirm Hutchinson and Clayton's claims with respect to the relative effects of reducing and non-reducing sugars on growth and cellulose decomposition by *S myxococcoides*, and to show that the same behavior was characteristic also of the other two species (table 1)

Like Imseneck (1941) I found a lesser degree of sensitivity to reducing sugars in the case of *S myxococcoides* than that reported by Hutchinson and Clayton Also noteworthy is the relatively greater resistance of *C rubra* than of the other two species The general purport of the results was quite clear however, and there could be no doubt that the prior observations on this anomalous behavior were correct The only question appeared to be what explanation to offer for them

At this stage it seemed of interest to determine whether reducing sugars exerted an effect on the respiration as well as on the growth of the cytophagas Consequently a Warburg experiment was set up to obtain information on this point

The species used was *C hutchinsonii* Cultures were grown at 30°C on a shaking machine in shallow layers of a liquid medium containing precipitated cellulose prepared according to the method of Kalnins (1930) It had been found that heavy cultures of all three species could be obtained in a few days under these conditions Due to copious slime formation, it is impossible to separate the cells of *C hutchinsonii* from a liquid medium by centrifugation, so 3-day-old cultures were simply centrifuged lightly to remove unattacked particles of cellulose and used as they were in the Warburg vessels Oxygen uptake by 2.0 ml amounts of such cultures was measured in the absence of sugar and with the addition of cellulose, cellobiose and glucose The amount of the two latter materials was 0.1 ml of a M/40 and of a M/20 solution respectively In the case of cellulose, 0.1 ml of a heavy suspension of the precipitated product was employed, the exact amount was not determined, since over the period of the experiment the quantity broken down would in any case be very small

The result of this experiment, as shown in the accompanying graph, was entirely unexpected Autorespiration was high, but the vessel containing cellulose showed an oxygen uptake which was appreciably greater Glucose and cellobiose, far from reducing autorespiration, caused an increase of oxygen uptake substantially the same as that with cellulose, a result most easily explicable on the assumption that these substances, like cellulose, were used by the bacteria in an oxidative process

Since it seemed most improbable that compounds which could be oxidized readily under the conditions of a Warburg experiment should be not only unutilizable but inhibiting in growth experiments, the previous work was examined critically for possible flaws. In all my growth experiments the sugars had been sterilized by autoclaving together with the mineral constituents of the medium, whereas the sugars used in the Warburg experiment were naturally not sterilized

TABLE 1

Growth at 30 C by *S. myxococcoides*, *C. hutchinsonii* and *C. rubra* in the presence of cellulose and varying percentages of heat sterilized glucose, sucrose and lactose

ORGANISM	SUGAR	TIME	PERCENTAGE OF SUGAR								
			0.005	0.01	0.025	0.05	0.1	0.25	0.5	1.0	2.0
<i>S. myxococcoides</i>	Glucose	days									
		6	4	4	4	4	4	0	0	0	0
	20	4	4	4	4	4	1	0	0	0	
	Sucrose	6	4	4	4	4	4	4	4	4	4
		20	4	4	4	4	4	4	4	4	4
	Lactose	6	4	4	4	4	4	4	0	0	0
20		4	4	4	4	4	4	2	0	0	
<i>C. hutchinsonii</i>	Glucose	6	4	4	4	4	3	0	0	0	0
		20	4	4	4	4	4	4	0	0	0
	Sucrose	6	4	4	4	4	4	4	4	4	0
		20	4	4	4	4	4	4	4	4	4
	Lactose	6	4	4	4	4	0	0	0	0	0
		20	4	4	4	4	4	0	0	0	0
<i>C. rubra</i>	Glucose	6	4	4	4	4	4	4	1	0	0
		20	4	4	4	4	4	4	1	0	0
	Sucrose	6	4	4	4	4	4	4	4	4	4
		20	4	4	4	4	4	4	4	4	4
	Lactose	6	4	4	4	4	4	4	4	1	0
		20	4	4	4	4	4	4	4	4	0

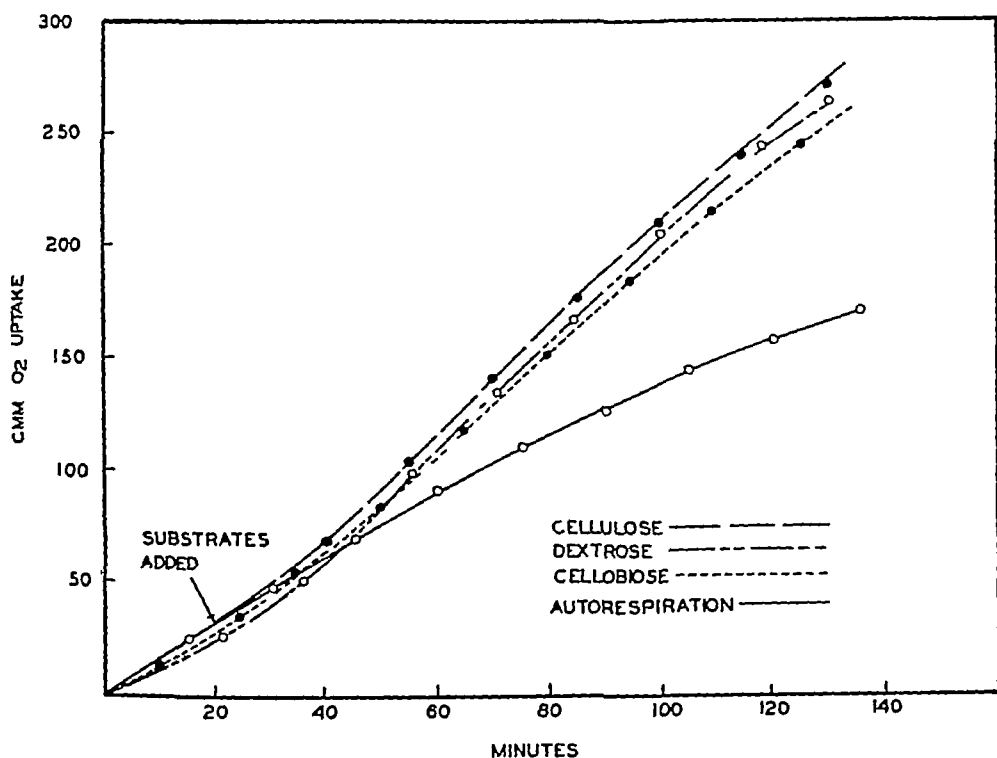
0 = no growth, 4 = maximum growth

at all. It has long been known that heating, especially in the presence of phosphates, causes some decomposition of reducing sugars ("caramelization") and, although the products formed are not generally considered harmful to bacteria, the effect of heat sterilization seemed the most likely cause for the discrepancies.

The growth experiments were accordingly repeated using glucose sterilized by filtration through Seitz filters and added to the autoclaved mineral base. Under such conditions, *S. myxococcoides*, *C. hutchinsonii* and *C. rubra* all grew excellently

over a wide range of glucose concentrations. Furthermore, the glucose sterilized by filtration exerted no "toxic effect" on growth in the presence of cellulose. With the highest concentrations of glucose tested there was, it is true, a decrease in cellulose decomposition, but at the same time abundant growth occurred in the surrounding liquid medium. This partial inhibition of cellulose decomposition is probably due to preferential utilization of the monosaccharide.

A re-examination of the carbon requirements of all three organisms using sugars sterilized by filtration yielded the results presented in table 2. *S. myxococcoides* and *C. hutchinsonii* were able to develop only at the expense of cellobiose and glucose in addition to cellulose. *C. rubra*, however, could utilize



OXYGEN UPTAKE BY *CYTOPHAGA HUTCHINSONII* IN THE PRESENCE OF GLUCOSE, CELLOBIOSE AND CELLULOSE AND IN THE ABSENCE OF ANY SUBSTRATE

also mannose and xylose. Thus the biochemical anomaly of "obligate" cellulose decomposition by the cytophagas has been disposed of finally. It must still be admitted that the range of available carbon sources for these organisms is somewhat narrow, indicating a considerable degree of specialization, however, results of a similar nature are not exceptional and have been reported for many other microorganisms. The cellulose-decomposing chytridiaceous fungus, *Rhizophlyctis rosea*, for example, grows well only with cellulose, cellobiose and glucose (Stamer, 1942a).

The optimum glucose concentration appears to vary with the different species. The results of experiments on this point, using liquid media, are given in table 3.

On solid media the behavior of the organisms with respect to sugar concentration is somewhat different. *S. myzococcoides* and *C. rubra* develop scantily, if at all, on mineral agar with 1.0 per cent glucose, although *C. hutchinsonii* grows abundantly. Development of the two former species on plates is regular only when the sugar concentration does not exceed 0.2 per cent. Possibly when filtered glucose is added to the melted mineral agar, which is at a temperature of about 50°C (such a temperature being necessary in order to allow time for the pouring of plates or slants before the agar hardens), sufficient decomposition of the sugar occurs to affect adversely the subsequent development of *S. myzococcoides* and *C. rubra*.

TABLE 2

The utilization of different carbon sources (concentration of 0.1 per cent) by *S. myzococcoides*, *C. hutchinsonii* and *C. rubra* after 5 days at 30°C

SUBSTANCE	<i>S. MYZOCOC- COIDES</i>	<i>C. HUTCHINSONII</i>	<i>C. RUBRA</i>
Control	0	0	0
Xylose	0	0	2
Arabinose	0	0	0
Galactose	0	0	0
Mannose	0	0	4
Levulose	0	0	0
Glucose	4	4	4
Cellobiose	4	4	4
Mannitol	0	0	0
Sodium pyruvate	0	0	0

0 = no growth, 4 = maximum growth

TABLE 3

Growth of *S. myzococcoides*, *C. hutchinsonii* and *C. rubra* with varying concentrations of glucose after 5 days at 30°C

ORGANISM	GLUCOSE, PER CENT						
	0.05	0.1	0.25	0.5	0.75	1.0	2.0
<i>S. myzococcoides</i>	3	3	4	4	4	4	4
<i>C. hutchinsonii</i>	2	2	3	4	4	4	3
<i>C. rubra</i>	2	3	4	3	3	3	3

0 = no growth, 4 = maximum growth

I have made no attempts to determine the nature of the toxic material formed during the heat sterilization of sugars. In view of the complexity of the chemical changes which occur during caramelization, and the inadequate characterization of the resultant compounds, this would probably be a difficult chemical problem. Nevertheless, it is an interesting subject for future investigation. The toxic material must be active in exceedingly low concentrations, glucose is not destroyed to an extent greater than five per cent during autoclaving, so that a (toxic) autoclaved 0.3 per cent glucose solution would not contain more than 0.015 per cent of total caramelization products.

When grown on agar plates, *S. myzococcoides* and *C. rubra* often effect a noticeable etching of the surface. The colonies become shallowly and irregularly

sunken into the surface of the medium, a phenomenon which is highly suggestive of agar decomposition. Nevertheless, all attempts to find supporting evidence for agar decomposition by these two species have been unsuccessful. Absolutely no development occurs on mineral agar plates devoid of glucose, even when the medium is heavily buffered with  $\text{CaCO}_3$  to counteract the acidity which might result from a breakdown of the agar. Gran's (1902) test<sup>4</sup> fails to show gelase fields. There is no obvious change in the consistency of the agar after growth, such as one invariably finds with agar-decomposers. Perhaps the most convincing evidence of all is the failure of these two species to develop at the expense of galactose, the chief constituent monosaccharide of agar. As a result of all these facts, one must conclude that the etching of agar surfaces by these two species is a purely physical phenomenon.

I have observed a similar, though less pronounced etching on old plates of several of the higher myxobacteria, it is particularly frequent in cultures of *Myxococcus virescens*. It is not improbable that this peculiar behavior of myxobacteria has been mistaken in the past for true agar decomposition. Yoshii (1926) claimed the existence of this property in *Myxococcus rubescens*, and both Beebe and Smieszko (personal communications) have suggested that agar breakdown characterized some of the myxococci with which they worked. I have never encountered a convincing case of agar decomposition among the higher myxobacteria, and in view of my experiences with *S. myxococcoides* and *C. rubra* it would perhaps be wise to treat these claims with a certain degree of skepticism, at least until some evidence more cogent than superficial appearance has been brought forward.

There thus seems good reason to ascribe a physical basis to the etching phenomenon, but what its actual mechanism may be is hard to say. Diatoms behave in a like manner when grown on agar plates, but this is due to the plowing of furrows by the siliceous walls of the cells as they creep over the surface. The delicate vegetative cells of myxobacteria, devoid of any kind of wall, could not conceivably bring about such a mechanical erosion.

#### *Nitrogen requirements and general conditions of growth*

The range of substances capable of serving as suitable nitrogen sources for growth of the soil cytophagas is shown in table 4. To be noted is the fact that peptone, yeast autolysate, aspartic acid and asparagin support development almost or quite as good as that which occurs with nitrate or ammonia. In general, results for the three species are very similar, following the same essential pattern. The only marked exception is the difference between *S. myxococcoides* and the amicrocystogenous species with respect to the utilization of urea. It is curious that the monoamino monocarboxylic amino acids (glycine, alanine and leucine) should be unutilizable, whereas both aspartic acid and asparagin provide good sources of nitrogen. None of the compounds tested was capable of serving as sole source of both carbon and nitrogen, apparently only the carbohydrates are suitable carbon sources for this group.

The data on nitrogen requirements for *S. myxococcoides* agree with those previously given for this species by Hutchinson and Clayton (1919) and

<sup>4</sup> This test, which gives unequivocal results with all agar-decomposers so far known, consists in flooding the surface of the plate with an I-KI solution, the attacked areas of agar surrounding the colonies take on at most a light straw color, whereas the undestroyed agar stains a reddish-violet.

Imseneckı and Solntzeva (1936) insofar as these workers tested the same compounds

The growth of the three species at different temperatures revealed clear-cut differences between the amicrocystogenous species and *S myxococcoides*, as shown in table 5

TABLE 4

*Growth of the soil cytophagas on cellulose in the presence of various nitrogen compounds*

SUBSTANCE	<i>S. MYXOCOC- COIDES</i>	<i>C. HUTCHINSONII</i>	<i>C. RUBRA</i>
Control (-N)	0	0	0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1%	4	4	4
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 1.0%	0	0	0
KNO <sub>3</sub> , 0.1%	4	4	4
KNO <sub>3</sub> , 1.0%	2-3	0	0
Urea, 0.1%	2-3	0	0
Glycine, 0.1%	0	0	0
Alanine, 0.1%	0	0	0
Leucine, 0.1%	0	0	0
Aspartic acid, 0.1%	1	3	3
Aspartic acid, 0.5%	3	4	4
Asparagin, 0.1%	2-3	2	2
Asparagin, 1.0%	3-4	2	4
Peptone, 0.1%	4	4	4
Peptone, 1.0%	2	2	2
Yeast autolysate, 1.0%	1-2	3	3

0 = no growth, 4 = maximum growth

TABLE 5

*Growth of S myxococcoides, C hutchinsonii and C rubra at various temperatures after 5 days and after 8 weeks (latter in parentheses) on a mineral, filter-paper medium*

ORGANISM	TEMPERATURE, C				
	15	22	25	30	35
<i>S myxococcoides</i>	0	1(3)	3	3	4
<i>C hutchinsonii</i>	0(2)	3	3	4	0
<i>C rubra</i>	0(2)	3	3	4	0

0 = no growth, 4 = maximum growth

All the soil cytophagas show a distinct preference for a neutral or alkaline environment, through the pH range from 6.5 to 8.5 (most alkaline tested) growth is equally good. At a pH of 6.0 or lower development ceases.

Imseneckı and Solntzeva (1936) have stated, in contrast to all previous workers, that the cytophagas are not strictly aerobic. Consequently I have examined this point with some care, but have been unable to substantiate their claim. It is true that some development takes place in cultures (particularly of *C rubra*) when the access of air is restricted, but

growth under such conditions is markedly slower than when full aeration is permitted. This is clearly shown, for example, by the very slow development on portions of filter paper which are even only slightly below the level of the liquid in flask cultures. Under conditions of *total oxygen exclusion* (cultures in bottles) growth ceases absolutely. It should be remembered that this is the real criterion of whether or not an organism is strictly aerobic. Slow development at a low oxygen tension may merely mean that organisms are carrying on their customary type of oxidative metabolism at a reduced rate. Growth is not possible under conditions of total oxygen exclusion even when nitrates are present, indicating that the cytophagas are incapable of employing denitrification as a respiratory mechanism for oxidative metabolism. This point is of some interest in view of van Iterson's (1904) finding that cellulose decomposition, resulting in slimy, yellow growth, can occur in enrichment cultures concomitantly with denitrification. From his description, it seemed possible that cytophagas might be developing under these conditions. However, the negative outcome of denitrification experiments with pure cultures makes it unlikely that this is actually the case. In a repetition of van Iterson's enrichment culture experiments for cellulose-decomposing denitrifiers I have found a predominating microflora of the *Pseudomonas* type.

In conclusion, a few words should be said about the possible beneficial effects of accompanying non-cellulose-decomposing bacteria on the development of the cytophagas. On the basis of careful and thorough experiments, Imseneck and Solntzeva (1936) have claimed that certain organisms, often found in impure cultures of *S. myxococcorides* and *S. ellipsospora*, greatly stimulate growth and cellulose decomposition by these two species when inoculated with them into a liquid mineral medium containing cellulose. They state that in certain cases *S. ellipsospora* does not grow at all in pure culture, whereas it invariably develops well with the accompanying bacteria. I have never observed a stimulatory effect of this nature with my soil cytophaga strains, which grow and decompose cellulose in pure culture just as well as when still accompanied by contaminating organisms.

Imseneck and Solntzeva, in discussing their results, pointed out that growth stimulation in mixed cultures is a common phenomenon among myxobacteria. In the higher myxobacteria, where it is often extremely marked, it is due to the ability to lyse cells of other microbes and develop on the nutrients thus released. This lysis, brought about by extracellular enzymes, is made evident by the formation of cleared areas around the swarm when myxobacteria are inoculated on an agar medium containing a suspension of susceptible cells, as first shown by Beebe (1941). None of the cytophagas which I have examined possess this property; they can neither lyse nor develop at the expense of other bacteria. The most probable explanation of Imseneck and Solntzeva's observations is that certain strains of the cytophagas have lost the ability to synthesize some growth factors, at least in the amounts necessary for maximal growth. Were this the case, accompanying bacteria might well stimulate growth and cellulose decomposition in a strictly mineral medium with cellulose by providing the critical growth factors in optimal quantity.

#### *The physical aspects of cellulose decomposition*

The cytophagas have been shown to attack a wide variety of cellulose materials, of which filter paper has been most generally employed. On this substrate

the majority of species exert a rapid and profound effect, causing a complete lysis of the fiber structure in a few days. In its place, there accumulates a mass of cells embedded in a slime which is often abundantly produced. Imseneckı and Solntzeva (1936) have used the expressive term "thawing" to describe the rapid lytic action on filter paper. While such a mode of destruction is characteristic of the *Sporocytophaga* species, of *C. hutchinsonii* and of *C. aurantiaca*, it never occurs in cultures of *C. rubra*. This species acts on filter paper in a manner reminiscent of the *Cellvibrio* group, the substrate is widely invaded and superficially attacked, but the actual fiber structure is only slowly and incompletely destroyed. The paper becomes pulpy and macerated, so that when shaken with water it breaks up at once into a large number of small fiber particles. The slime production characteristic of the other soil species is also very much reduced. The difference between lytic and macerative decomposition, although sharp and clear-cut when filter paper is used as a substrate, tend to disappear when the organisms are grown on some other materials.

Krzemienievska (1933) and Imseneckı and Solntzeva (1936) have reported good development on cellophane. My experience with this substrate has been less satisfactory. *S. myzococcoides*, *C. hutchinsonii* and *C. rubra* will all grow on strips of cellophane in a liquid medium, but the actual dissolution of the cellophane is slow and incomplete. I could obtain no growth on cellophane laid on agar plates, the upper surface of the material dries out rapidly and the organisms are unable to gain a foothold. Perhaps the differences of opinion as to the value of this material are due to the use of differently prepared cellophanes.

Growth on precipitated cellulose ("hydrocellulose") is instructive. All species will develop abundantly on this material provided that certain physical factors are taken into account. The experiences of Jensen (1940) and myself have shown that attack on precipitated cellulose in agar is only possible when the organisms can move through the agar. As mentioned earlier, development on cellulose agar plates is never accompanied by the formation of enzymatic fields of cellulose decomposition around the growth, as is the case with most other aerobic cellulose decomposers (e.g., the vibrios, the *Cellulomonas* group, the cellulose-decomposing bacilli and *Rhizophlyctis rosea*). This provides strong support for the contention of Winogradsky and others that *the cytophagas must be in direct contact with cellulose in order to attack it*.

In liquid media, growth with precipitated cellulose is extremely rapid when cultures are kept agitated to maintain the cellulose in suspension and provide good aeration.

A noteworthy phenomenon is the excellent development of the cytophagas on pellicles from *Acetobacter xylinum* cultures. *Acetobacter xylinum* is one of the very few bacteria able to synthesize cellulose, which is produced in the form of a firm, greyish-white pellicle on the surface of culture media. The pellicle gradually increases in thickness as a culture ages until it may attain a depth of as much as a centimeter. When such pellicles are successively washed in water, alcohol and ether and then dried, they have the appearance of parchment sheets. Strips of this material are rapidly and completely destroyed by the cytophagas. A drop of water to which attacked portions of the pellicle are added becomes heavily turbid owing to the release of millions of *Acetobacter* cells previously imprisoned within the cellulose.

For the examination of the microscopic aspects of cellulose decomposition, I have found cotton wool to be a superior material. The cytophagas which provoke a rapid lytic destruction of filter paper attack cotton fibers more gradually, so that one has a better opportunity to observe the course of breakdown. Cotton fibers are longer and more regular than the fibers in filter paper. Finally, much work (see e.g., Kerr and Anderson, 1938) has been done on the structure of the cotton fiber and its micellar orientation, making an interpretation of the manner of destruction easier. For most of the work discussed in the following paragraphs *C. hutchinsonii* and *C. rubra* have been used. *S. myxococcoides* differs hardly at all from *C. hutchinsonii* in its mode of attack on all kinds of cellulose, and is not a satisfactory form to use for microscopic studies because the microcysts impede clear observation of vegetative cell orientation.

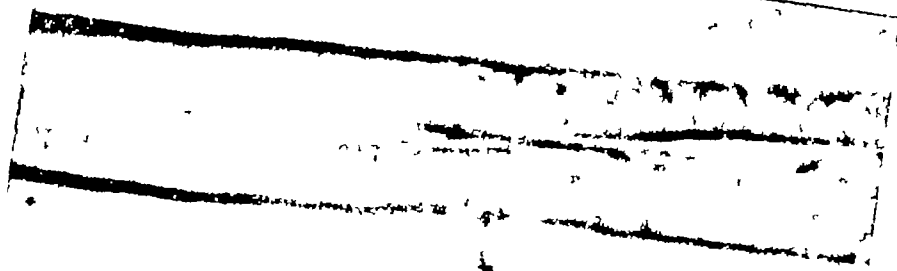
The differences between the lytic decomposition by *C. hutchinsonii* and the macerative decomposition by *C. rubra*, so pronounced on filter paper, are less apparent when cotton wool is used. Cotton fibers, even when heavily invested by *C. hutchinsonii*, retain to a considerable degree their original structural and tensile properties.

The primary attack by both *C. hutchinsonii* and *C. rubra* is predominantly external. There is only very rarely an invasion of the lumen by *C. rubra*. Although this phenomenon is more frequent in the case of *C. hutchinsonii*, it occurs even with this species in a small minority of the fibers. As the age of the culture increases, lumen invasion becomes more frequent, taking place both by entry of the organisms at one end of the fiber and by a breaking through from some previously attacked surface region.

Attacked fibers have a characteristically mottled or etched appearance under the microscope. Closer examination shows that the originally smooth surface has been eaten away in a more or less regular manner. The real nature of the change becomes clear when the material is treated with concentrated NaOH, which causes a shortening and swelling of the fibers. After such treatment, normal fibers have a perfectly smooth and even surface as shown in fig. 13. Attacked fibers, on the other hand, are covered with regular striations as shown in figs. 14 to 16. These striations consist of closely approximated spiral furrows which girdle the fiber at a steep though varying angle to the long axis.

The swelling treatment, while revealing something of the pattern of external decomposition, destroys the arrangement of the cytophaga cells. Consequently, it was decided to grow the organisms on pre-swollen material. Cotton wool was placed in 15 per cent NaOH for about five minutes and then thoroughly washed to remove the alkali. When grown on such swollen fibers, the cytophagas produce spiral furrows similar to those demonstrable by swelling after attack. Not infrequently, these furrows run in two systems of crossed spirals. By staining according to Winogradsky's method, it is possible to show that many of the attacking cells lie in these furrows, oriented in the same direction as the furrow itself. Unfortunately, it is not easy to take convincing photomicrographs of this, but figs. 17 to 24 give some idea of the appearance.

As the organisms penetrate inward their orientation often changes, and they



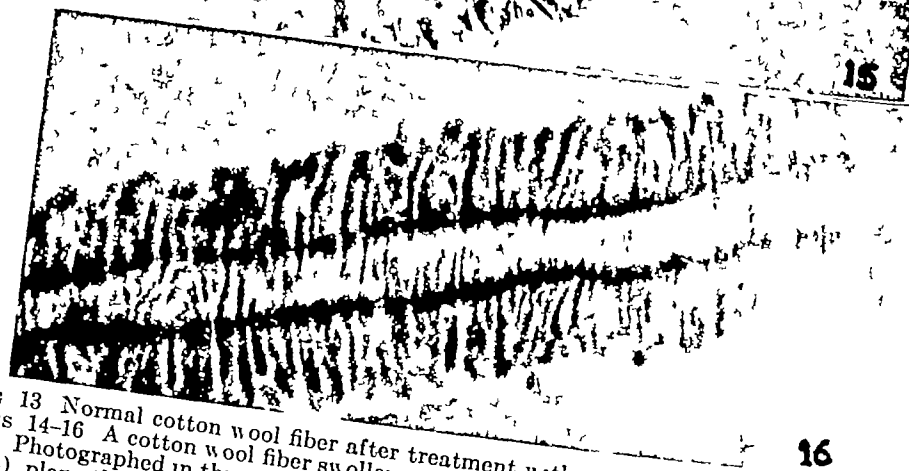
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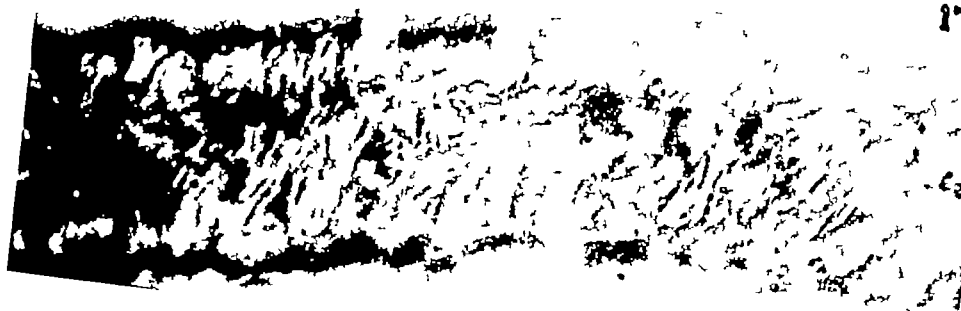
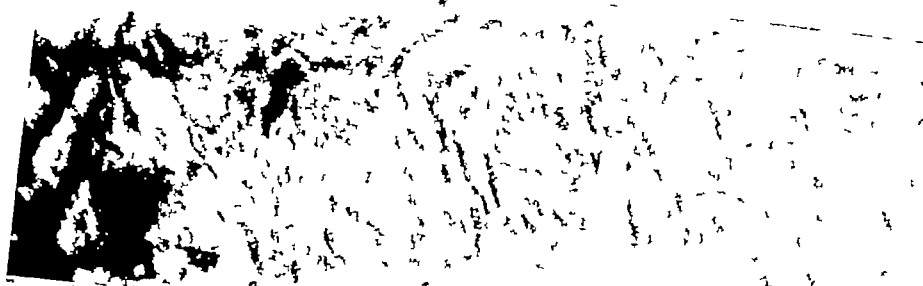


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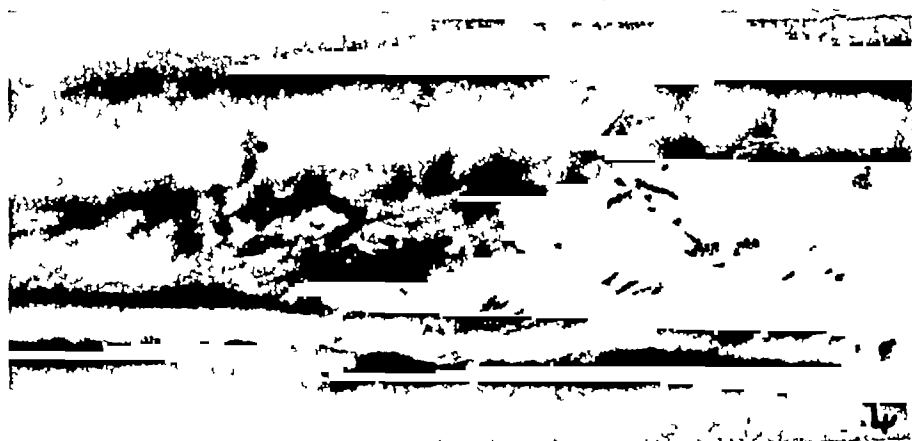
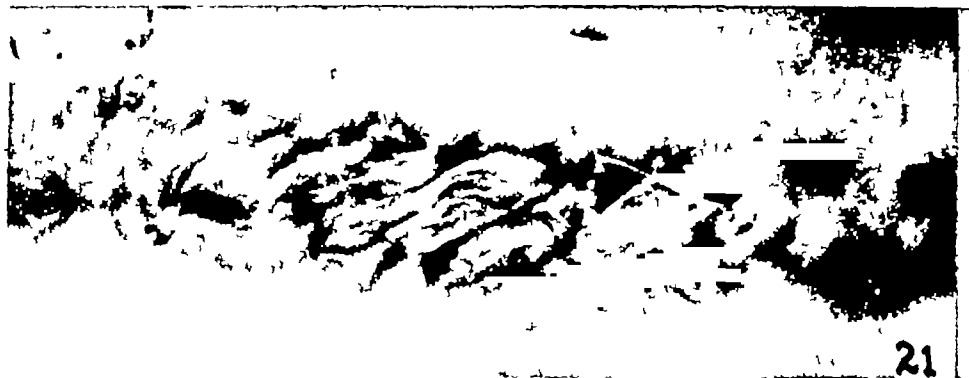


16

Fig 13 Normal cotton wool fiber after treatment with concentrated NaOH  
 Figs 14-16 A cotton wool fiber swollen with NaOH after being attacked by *C. hutchinsonii*. Photographed in three planes to show spiral pattern of disintegration, lower surface (fig 14), plane through lumen (fig 15) and upper surface (fig 16)



Figs 17-20 Attacked cotton wool fibers, swollen with concentrated NaOH before inoculation Winogradsky's stain  $\times 1000$  approx  
Figs 17 and 18 show the upper and lower surfaces respectively of a single fiber Figs 19 and 20 show invasion of the lumen accompanying external decomposition



Figs 21-24 Attacked cotton wool fibers, swollen with concentrated NaOH before inoculation, showing the relation of the cytophaga cells to the pattern of decomposition Winogradsky's stain  $\times 950$  approx. In fig 24 the indentations on the cellulose where cells have previously lain can be seen

generally lie in spirals which run less nearly at right angles to the long axis of the fiber. In some cases the decomposition of the substrate is still evidenced by the formation of furrows, but the attack often seems to become more generalized and even

However carefully stained preparations are made, many cells become loosened and detached from the fibers. Where this occurs in a region originally well covered with cells, it is not infrequently possible to discern minute indentations on the surface of the cellulose which, from their size and orientation, seem to be places where bacteria previously lay and dissolved away the underlying substrate (fig. 24). Cells remaining attached in such regions are of approximately the same size and oriented in the same manner as the indentations.

How do the known facts about the structure of the cotton fiber fit in with these observations? The cellulose is known to be deposited in the form of successive concentric layers, in each of which there is a definite micellar orientation following the pattern of a spiral with frequent reversals. Thus it seems not unreasonable to assume that the spiral furrows observable in the attacked fibers do actually follow the orientation of the cellulose micelles. Nor is this difficult to understand when one remembers two things, the elasticotactic behavior of myxobacteria and the localized action on cellulose of the cytophagas. The cells which first reach the surface of a cellulose fiber will be oriented by and move parallel to the underlying micellar structure, while their attack will be confined to the substrate in their immediate neighborhood. In this way it is readily conceivable that an intense decomposition may take place proceeding along a narrow spiral path, while neighboring regions of the fiber remain undamaged during the initial phase of decomposition. As growth proceeds, however, the cells will become spread out over and eventually cover the entire surface, so that the deeper layers undergo a more uniform and rapid breakdown.

#### *The chemical aspects of cellulose decomposition*

For many years it has been generally accepted that cellulose decomposition is brought about by two enzyme systems, cellulase and cellobiase. The former is presumed to hydrolyze cellulose to the disaccharide cellobiose, while the latter splits the cellobiose thus formed into glucose, which is absorbed by the cell. This pathway was first proposed by Pringsheim (1912) as a result of his studies on cellulose decomposition by a crude culture of thermophilic organisms. Pringsheim was able to demonstrate that by arresting development of the bacteria in various ways cellobiose and glucose could be made to accumulate in the medium. Since that time, his conclusions have received confirmation through the work of Karrer *et al* (1924, 1926, 1928) with cellulose-dissolving preparations from the gut of the snail, as well as through the work of Kalnins (1930), Simola (1931) and Pochon (1935) using bacteria, and of Vaitiovaara (1935) with fungi. In general, these investigators have confirmed the accumulation of reducing substances (identified either as cellobiose or as glucose or as both) in cellulose-decomposing cultures, and have established (Karrer *et al*, Simola) the cellulolytic activity of cell-free extracts.

On the basis of Pringsheim's formulation it is logical to conclude that cellulose decomposing organisms possess the ability to attack and grow at the expense of cellobiose and glucose. Yet all previous workers who have studied the cytophagas have been faced with the unpleasant fact that these organisms acted apparently as "obligate" cellulose-decomposers, whose growth was inhibited or stopped by very small amounts of glucose. How was this dilemma to be resolved?

Winogradsky (1929) ignored the Pringsheim formulation of a simple hydrolytic route and developed an elaborate theory of oxidative decomposition which he considered to hold for cellulose breakdown by all aerobic bacteria. Since Winogradsky's theory has received some measure of subsequent attention and support, it seems desirable to consider his data and conclusions in some detail.

Winogradsky observed that filter paper attacked by aerobic bacteria reacted in a manner which indicated that it was similar in certain respects to the rather ill-defined material known as oxycellulose, which results from a partial chemical oxidation of cellulose. Attacked portions of filter paper were claimed to have acidic properties, as shown particularly by their intense staining with methylene blue, a basic dye. The "oxycellulose," dispersible in water and soluble in weak bases, could be extracted from the attacked fibers, leaving behind the unchanged cellulose. Unlike chemically produced oxycellulose, it possessed no reducing power, but this was regarded by Winogradsky as marking merely "une différence essentielle entre l'oxydation biologique et l'oxydation chimique." No notable end-products of any other sort (organic acids, alcohols) were discovered in cellulose cultures, although Hutchinson and Clayton had claimed that *S. myxococcorides* produced small amounts of butyric acid. On this point Winogradsky's comment (fully substantiated by all later workers) was "Il n'y a production d'aucun acide gras, ni généralement d'aucun produit volatil. Le fait est tellement régulier et constant que la présence de ces produits est à envisager comme un indice de l'ingérence des microbes anaérobies."

The conclusion which Winogradsky drew from these observations was that the cellulose underwent a direct attack through the oxidation of the protruding carbinol groups to aldehydic and ultimately to carboxylic groups, with the resultant formation and accumulation of "oxycellulose." Curiously enough, he made no mention of the peculiarities in carbon requirements of the cytophagas, although one might have imagined that this was the factor which really appeared to make necessary such a drastic abandonment of the conventional view of cellulose decomposition.

The characteristics of the supposed "oxycellulose" were left extremely vague. *Cytophaga hutchinsonii*, the species among the cytophagas which Winogradsky studied most carefully, always gives rise in cultures to an extreme abundance of a colloidal, mucilaginous material. Subsequent workers have taken it for granted that this was the material which Winogradsky considered to be the typical oxycellulose. However, although he referred repeatedly to the "gel organique" or "colloïde organique" produced by *C. hutchinsonii*, it is by no means clear that such was the case, and in several places a distinction is drawn between the two. The cellvibrios never produce a gummy material when acting on cellulose, yet they too, according to Winogradsky, oxidize the cellulose to oxycellulose.

Winogradsky's theory has been accepted by Loicjanskaia (1937) and by Walker and Warren (1938). The latter authors, again using *C. hutchinsonii*, isolated the mucilaginous material and studied it chemically. They were able to show that it was a polyuronide, and concluded that it was an "acidic type of oxycellulose" formed from the partial breakdown of cellulose.

Imseneck and Solntzeva (1936) were the first to perceive some of the flaws in Winogradsky's theory. They discovered that attacked filter paper was stained just as heavily by acidic as by basic dyes, and from this concluded very reasonably that it was the accumulated bacterial cells, rather than any "oxycellulose," which actually absorbed and retained methylene blue. Furthermore, they pointed out that the "gel organique" in cytophaga cultures need not have resulted directly from the cellulose, but could equally well be a synthetic product of the cells themselves.

Norman and his associates (Norman and Bartholomew, 1940, Norman and Fuller, 1942) have also criticized the oxidative theory. They state that such an oxidative attack presupposes the existence of an oxidative exo-enzyme system, which is highly improbable. Furthermore, no energy could be obtained by the cells until water-soluble fragments had been produced, so that there is no good apparent reason why oxycellulose should accumulate. They further disagreed with Walker and Warren's characterization of the mucilaginous material as an oxycellulose, and reported it to be a microbial gum containing both uronic and pentose groups. Thus the factual basis of the oxidative theory has become, to say the least, highly doubtful.

The discovery that the soil cytophagas can in fact attack and grow at the expense of cellobiose and glucose has now removed even the *raison d'être* of this theory, and there is thus every reason to consider that a hydrolytic or phosphorolytic breakdown of the type suggested by Pringsheim is the actual mechanism. A decisive confirmation of the contention made by Imseneck and Solntzeva and Norman and Bartholomew that the mucilage produced by the cytophagas is a microbial gum, is provided by the manner of growth on glucose. *Cytophaga hutchinsonii*, which produced mucilage to a greater degree than any other species, will do so just as readily when growing on glucose as when growing on cellulose. In fact, liquid glucose cultures of this organism may become so viscous that they are hard to pipette. The material can be precipitated from such cultures by alcohol or acetone, and exhibits all the properties which were reported by Winogradsky and by Walker and Warren as characterizing oxycellulose. Were the material really an oxycellulose, it should of course be produced only as a result of growth on cellulose.

Nevertheless, one further piece of evidence in favor of a breakdown via cellobiose and glucose has yet to be produced. One might expect that under certain conditions reducing sugars would accumulate in cellulose cultures, yet they have never been reported by previous workers. I have repeatedly tested normal cultures without ever finding a trace of reduction. While this is perhaps not unexpected, attempts to obtain evidence for the accumulation of reducing sugars under unfavorable conditions have also been uniformly unsuccessful. Vigorous

cultures of *C. hutchinsonii* and *C. rubra* have been treated with toluene for as long as a fortnight with negative results. Similar experiments in which anaerobiosis or maintenance at a supramaximal growth temperature (45 C) were used instead of the toluene treatment were likewise without success. It is not easy to offer a reasonable explanation of these findings, particularly in view of the ease with which various workers (e.g., Kalnins) have demonstrated the accumulation of reducing sugars in cellulose cultures of other cellulose-decomposing bacteria. However, one should recall that the cytophagas differ markedly from most aerobic cellulose-decomposers through their inability to decompose cellulose except in the close neighborhood of the cells.

Does this perhaps imply that the cytophagas split cellulose by a mechanism different from that employed by other aerobic bacteria? Organisms like the cellvibrios clearly bring about an hydrolysis of the cellulose molecule, their cellulase acts on cellulose in the same sort of way as amylases act on starch and glycogen. It has become clear recently through the work of Cori *et al.* and of Hanes (for references see Cori and Cori, 1941) that starch and glycogen can also be decomposed by an entirely different enzyme system, phosphorylase, which causes a phosphorolytic rather than an hydrolytic fission of the polysaccharide. Offhand it does not seem improbable that a phosphorolytic decomposition of cellulose may also take place. Were such the case in the cytophagas, continued metabolism might be necessary for the maintenance of the phosphate cycle, and hence treatments designed to inhibit normal respiration would be likely to inhibit cellulose decomposition as well.

There is another possible explanation for the non-accumulation of reducing sugars which should be considered. The fission of a long-chain molecule like cellulose may be accomplished in one of two ways. It can take place either by the continued lopping off from the end of the chain of short terminal sections containing from one to a few glucose units, or by an approximately central splitting of the chain repeated on the resulting fragments with the production of shorter and shorter lengths. In the former case, a small amount of enzyme should give rise to detectable amounts of reducing sugars. In the latter case, however, even a large quantity of the enzyme would yield only very small amounts of reducing sugars when acting on a highly polymerized cellulose. If the cytophagas attack cellulose by this second method, the difficulties in finding reducing sugars are understandable.

To sum up, we may say that the evidence favors very strongly a decomposition of cellulose by the cytophagas proceeding by hydrolysis or phosphorolysis with the ultimate production of reducing sugars which enter the cell and there undergo an oxidative decomposition whose sole products are carbon dioxide and cell material.

#### PHYSIOLOGY OF THE MARINE CYTOPHAGAS

The properties of the marine cytophagas have been thoroughly discussed in two recent papers (Stanier, 1940, 1941) so that little need be said concerning them here. *C. diffluens* and *C. krzemienskiae* are agar-decomposers *par excellence*. Owing to their rapidly spreading growth (swarm extension) the surface of a plate culture becomes covered with organisms in a few days, and the

gelase fields, as shown by Gran's reaction, rarely extend beyond the borders of growth. However, the underlying agar becomes sunken and softened, and may ultimately be liquefied. Due to evaporation, liquefaction of the agar is often not apparent on plates, agar slants, on the other hand, become almost completely liquid after a fortnight. Considerable acid is produced during agar decomposition, and since the organisms grow well only in a neutral or alkaline medium, it is necessary to buffer agar-containing media with  $\text{CaCO}_3$  in order to obtain good growth and decomposition. The acid production is probably caused by the release of sulfuric acid from the agar, in which there are sulfuric ester linkages. Acid production from agar is also characteristic of other rapid agar-liquefiers (e.g., *Vibrio agar-liquefaciens*, *V. granii*), but not of the agar-softening types.

Both *C. diffluens* and *C. krzemieniewskae* attack a wide range of carbohydrates in addition to agar, as shown in table 6. Unlike the soil cytophagas, they are

TABLE 6  
Utilization of carbohydrates by *C. krzemieniewskae* and *C. diffluens*

SUBSTANCE	C. KRZEMIENIEWSKAE	C. DIFFLUENS
Arabinose	—	—
Xylose	+	+
Glucose	+	+
Galactose	+	+
Maltose	+	+
Sucrose	—	—
Cellobiose	+	+
Cellulose	+	+
Starch	+	—
Alginate acid	+	+
Chitin	—	—
Agar	+	+

not inhibited by the decomposition products resulting from the heat sterilization of reducing sugars.

It will be noted that both species attack cellulose. The decomposition is of the macerative type, and takes place extremely slowly in comparison with the action of the soil cytophagas. Strips of filter paper are disintegrated at the air-water interface after approximately ten days. The marine cytophagas can also obtain their carbon and energy requirements from proteinaceous materials, in marked contrast to the soil forms. Both species grow well with peptone or yeast extract in the absence of carbohydrates.

The only substances found to satisfy the nitrogen requirements of the marine cytophagas are yeast extract and peptone. There is absolutely no development in media containing a suitable carbohydrate when nitrates, ammonia, asparagin or any one of a variety of amino-acids is present as sole nitrogen source. This would appear to indicate that the marine cytophagas require preformed growth factors, amino-acids, or both.

The organisms are strictly aerobic. The temperature range for growth lies between 15 and 30 C. While this appears much the same as that of the amicrocystogenous soil species, the optimum for the marine species is appreciably lower, between 22 and 25 C.

In common with most marine bacteria, *C. diffluens* and *C. krzemienskiae* are obligately halophilic, failing to grow in media with a salt concentration below 1.5 per cent. The optimum salt concentration range for these forms is 2.0 to 4.0 per cent.

#### TAXONOMY

##### *Evidence concerning the systematic position of the cytophagas*

There are few examples which illustrate more clearly the weaknesses and uncertainties of bacterial classification in its broader aspects than the confusion which has surrounded the systematic position of the cytophaga group. These organisms were placed among the true bacteria, the spirochaetes and the actinomycetes before their relationships to the myxobacteria were finally recognized, and this in spite of the fact that *S. myxococcoides*, the species most studied, is morphologically a typical representative of the *Myxococcaceae*.

Van Iterson (1905) and Merker (1912), who observed the microcysts but not the vegetative cells of *S. myxococcoides*, considered it to be a member of the *Micrococcaceae* (i.e., an eubacterial form).

Hutchinson and Clayton's (1919) elucidation of the life cycle of *S. myxococcoides* showed clearly that the previous interpretations were incorrect and that the "coccus" forms represented the reproductive stage of an organism whose vegetative cells were rod-shaped. Furthermore, these workers realized that certain properties of the vegetative cells, such as their extreme flexibility, were incompatible with a position among the true bacteria. Unfortunately, they based their deductions as to the morphology of *S. myxococcoides* on the study of stained preparations which, due to the imperfect techniques employed, contained many "spirochaetal" artefacts. These were taken to represent a normal condition of the vegetative cells, and consequently Hutchinson and Clayton tentatively placed the organism in the *Spirochaetales* under the name *Spirochaeta cytophaga*.

Winogradsky (1929), as a result of his studies on the amicrocystogenous cytophagas, pointed out the incongruity of classing these forms with the spirochaetes, stressing particularly the absence of the highly characteristic active spirochaetal mode of locomotion. For this reason he placed them in the new genus *Cytophaga*, but made no suggestion as to its systematic position.

At about the same time Bokor (1930) concluded that "*Spirochaeta*" *cytophaga* had a life cycle in which there occurred a branched, mycelial stage. On this he based the suggestion that the organism was a representative of the *Actinomycetales*, among which he proposed to place it under the name *Mycococcus cytophagus*. As mentioned earlier, there can be no doubt that Bokor was dealing with a mixed culture of an actinomycete and a (probably) non-microcyst-forming cytophaga, hence his systematic deductions merit no further consideration.

In the fourth edition of Bergey's Manual of Determinative Bacteriology, Winogradsky's *Cytophaga* group was placed among the *Actinomycetales*, together with Winogradsky's other genera of cellulose-decomposing bacteria, *Cellvibrio* and *Cellfalcicula*. In the fifth and latest edition (1939) the two latter genera found their correct position in the *Pseudomonadaceae*, while the genus *Cytophaga* was tentatively removed to the *Spirochaetales* where it appeared as an appendix to the genus *Spirochaeta*.

The work of Krzemieniewska (1930, 1933) provided the first approach to a correct understanding of the systematic position of the cytophagas. Her careful study of the life cycle of "*Spirochaeta*" *cytophaga* revealed clearly the nature of the microcysts and their great resemblance in structure and mode of formation to the individual resting cells in the *Myxococcaceae*. This was recognized and stressed by Krzemieniewska, but particularly because the amicrocystogenous cytophagas did not appear to fit into the orthodox concept of myxobacteria, she was not at that time prepared to place them without reservation in the *Myxobacteriae*, although recognizing that this was a future possibility.

Krzemieniewska's conclusions were strengthened and extended by the work of Imsenecki and Solntzeva (1936), who discovered a second microcystogenous species in which they were able to show again a myxococcoid sequence of development. They confirmed the earlier observations of Stapp and Bortels (1934) on the creeping method of locomotion, the taxonomic significance of which they understood.

My own studies on the marine cytophagas (1940) showed the myxobacterial nature of the non-microcyst-forming members of the group, of which these were the first representatives to be obtained and studied in pure culture. The fact that they could develop on peptone-agar made possible a study of their mass growth and movement which could not at that time be undertaken with the soil forms. This study revealed yet another specific myxobacterial character in the cytophagas—colony increase by swarm extension. More recently, as discussed in a previous section, the growth of the soil cytophagas on glucose-agar has revealed the existence of swarming in some of these species also.

We are thus confronted with several different lines of evidence which, taken in conjunction, make it necessary to regard the cytophaga group as myxobacteria. mode of formation and structure of microcysts, mode of locomotion, lack of rigid cell walls, cell division by constriction, manner of colony growth.

#### *A survey of the higher myxobacteria and their relations to the cytophaga group*

It becomes our next task to consider the position which the cytophagas occupy among the higher myxobacteria.

Since their first recognition as an independent group by Thaxter (1892), the higher myxobacteria have been studied far more extensively by cryptogamic botanists than by bacteriologists, a fact of no little consequence from the standpoint of our present knowledge about them. Pure culture methods have been little used, and in general these organisms have been treated more as objects of mor-

phological interest than as biological entities. We know hardly more of some species than the final structure of their fruiting bodies.<sup>5</sup>

The higher myxobacteria have a life cycle which is divided into two sharply distinct phases. In the vegetative stage they occur as primitive, unicellular, rod-shaped organisms superficially not dissimilar from true bacteria. Each of the vegetative cells is capable of leading an independent existence. However, under the influence of as yet unknown stimuli, they come together and enter into a joint reproductive phase, during which thousands of individuals coöperate in the building of fruiting bodies which in some species are structurally quite complex. Particularly to be noted is the fact that many cells in a developing fruiting body may be used up in the production of supporting structures or protective membranes, thus losing their individual chances for survival while contributing to the reproduction of other cells. Throughout the whole range of living organisms, the only other known example of this type of development is provided by the *Acrasieae*, a small group of amoeboid protists.

Can one legitimately describe such organisms as "unicellular?" To do so, the term must be stretched beyond its accustomed meaning. It is not so much the size, complexity and many-celled nature of the fruiting structure, as primarily the existence in it of both reproductive and non-reproductive cells which marks it as beyond the unicellular level of organization. On the other hand, the vegetative phase of the myxobacteria and *Acrasieae* is typically unicellular, which makes it impossible to fit either group into the customary category of multicellular organisms. Unfortunately, biologists in general have paid very little attention to these assemblages, and consequently their theoretical biological interest has been overlooked.

It seems to me that we have to deal here with two exceptionally clear and complete, parallel, convergent series in which there has been an attempted evolution from unicellularity towards multicellularity along a path entirely different from that followed elsewhere in the *Monera* and *Protista*. In other groups, such as the *Volvocales*, where we can still trace by inference the probable evolutionary sequence which led to multicellular organisms, this development appears to have taken place by the continued association of vegetative cells after division, with the consequent formation of permanently many-celled, filamentous, spherical or dendritic structures. In the *Myxobacterales* and *Acrasieae*, however, the vegetative cells retained their individuality throughout the major portion of the life cycle, and the increase in complexity has occurred through the development of many-celled structures for reproduction and dispersal, produced by the aggregation of originally separate and spatially separated vegetative cells.

These two small assemblages obviously represent terminal evolutionary series, which failed to develop further and attain a distinctively multicellular level of

<sup>5</sup> Indicative of the botanical approach used in studying myxobacteria is the attention paid by most workers, from the time of Thaxter on, to the geographical ecology of these organisms. Papers with such titles as "Die Myxobakterien der Umgebung von Wien" or "Die Myxobakterien von Polen" come as somewhat of a shock to a bacteriologist, who accepts without hesitation the view that bacteria are of world-wide distribution.

organization The reasons for such a failure seem evident As long as the vegetative cells remained separate individuals, an augmentation in the size and complexity of the fruiting structure would decrease the opportunities for a successful termination of the fruiting process under the competitive conditions of a natural environment There are two decisive factors which limit the extent of fruiting development the number of vegetative cells which can be concentrated over a small area, and the time necessary for the completion of the process Short of a permanent union of the cells during the vegetative phase of the life cycle, which did not take place,<sup>6</sup> further evolutionary progress along the path begun in these groups is difficult to envisage

Nevertheless, the *Myxobacterae* and *Acrasieae* have continued to exist and are today of widespread occurrence, indicating that the communal fruiting process has had a definite survival value It permits a dispersal of the species far more extensive than is possible in the case of non-fruiting organisms with a similar vegetative morphology

It seems desirable to propose a name for the type of organization exemplified by these forms, and I should like to suggest the term *reproductive communalism*

Recognition of the higher myxobacteria has rested entirely on fruiting body structure and organization, and the whole group concept has been founded on the existence of a life cycle of the sort outlined above Does the absence of such a cycle in the cytophagas then exclude them from the myxobacteria or mark them as "aberrant" forms? Not at all! The remarkable phenomenon of fruiting body formation together with the methods of study employed has tended to obscure other less obvious, but taxonomically even more important, characters of the myxobacteria, namely, the properties of the individual vegetative cell These properties are of necessity brought to the fore in the cytophagas, but are equally evident, if looked for, in the higher myxobacteria These characters are not clearly presented in Jahn's (1924) definition of the class *Polyangidae* (*Myxobacterae*), which is the generally accepted one at present It reads as follows

"Der Vegetationskörper ist der Schwarm, der aus winzigen langgestreckten Stäbchen besteht Sie führen einen carotinartigen Farbstoff und bewegen sich gemeinsam in langer Front durch Schleimabsonderung fort Fruchtkörper entweder aus zahlreichen Sporen bestehend, die durch Verkürzung der Stäbchen entstehen, oder aus Cysten, in deren Inneren mehr oder minder verkürzte Stäbchen liegen "

Apart from its omissions, the above definition contains a statement concerning fruiting-body formation which makes it impossible to include the cytophagas, because they are chiefly defined by the absence of such structures, forming at best microcysts Yet the obvious morphological affinities between the cytophagas and the higher myxobacteria make it necessary to create one large taxonomic unit which will include both groups and which must, consequently, be based entirely on the structure of the vegetative cells

<sup>6</sup> Unless one chooses to regard the myxomycetes, with their plasmodial vegetative phase, as being derived from the *Acrasieae*, most competent authorities, however, consider that they evolved from a different group among the amoeboid protists

Two different procedures can be followed. The first is to maintain Jahn's definition of the class *Myxobacteriae* as restricted to the higher myxobacteria, and to create a corresponding class for non-fruiting organisms with like vegetative morphology. The two classes could then be combined in a new division. As an alternative solution, the class *Myxobacteriae* can be redefined to permit the inclusion of the cytophagas.

It would be difficult to justify the creation of a class for the cytophagas, since the separation of the two classes would rest on the presence or absence of fruiting bodies alone, which is an indefensible criterion for segregations of this magnitude. Therefore the second approach has been adopted, the class *Myxobacteriae* has been redefined chiefly on the basis of vegetative morphology (Stanier and van Niel, 1941). Besides making possible the inclusion of the cytophagas, it serves the useful purpose of accentuating the basic morphological distinctions, previously not sufficiently recognized, between the classes of *Myxobacteriae* and *Eubacteriae*. The definition is as follows.

#### Class *Myxobacteriae*

In the vegetative stage the organisms are rod-shaped. They do not possess rigid cell walls. Cell division occurs transversely, by constriction rather than by fission. Motility is never flagellar, it requires the presence of a substratum, and is of the creeping type found in the *Cyanophyta*.

Jahn, who developed the first and only detailed systematic treatment of the myxobacteria, recognized four families on the basis of the fruiting structures. His primary division was made on the shape of the microcysts, in the *Myxococcaceae* they are spherical or oval, whereas in the remaining families they are shortened rods. The *Archangiaceae* form fruits in which these shortened rods accumulate in irregular masses held together with hardened slime but never enclosed within an envelope or cyst. In the *Sorangiaceae*, the rod-shaped microcysts are enclosed within small, angular cysts (the secondary cysts) which are often held together by still larger envelopes (the primary cysts or sori). In the *Polyangiaceae* the microcysts occur in cysts which are usually larger than the secondary cysts of the *Sorangiaceae* and invariably rounded.

Since Jahn's work, only one major change has been suggested. As a result of extended studies on the vegetative cell throughout all groups of the higher myxobacteria, the Krzemieniewskis (1928) have laid down the generalization that two quite distinct cell shapes occur. In the *Sorangiaceae* and some species of the *Archangiaceae* one finds short, thick rods with blunt, almost square ends, whereas in the remaining groups (*Myxococcaceae*, *Polyangiaceae* and certain *Archangium* species) the vegetative cells are long, slender rods, often somewhat spindle-shaped with pointed tips. Furthermore, these workers (Krzemieniewski, H and S, 1937) found that on long-continued cultivation some *Sorangium* species lose the ability to form well-defined cysts and thus become indistinguishable from the archangia. It is clearly undesirable to base the separation of families on a character which is capable of changing in this way, and the Krzemieniewskis have suggested that the family *Archangiaceae* should be abolished. Its constituent species can be distributed between the *Sorangiaceae* and the *Polyangiaceae* on the

basis of the vegetative cell shape, which would then be the distinguishing character between these two families. My own studies on the higher myxobacteria (unpublished) have convinced me of the soundness of this proposal. Hence it will be adopted here.

How may the cytophagas be brought into this system? On the basis of cell shape, they are clearly related to the *Myxococcaceae* and *Polyangriaceae*, a conclusion which is further strengthened by the typically myxococcoid nature of the microcysts in *S. myxococcoides* and *S. ellipsospora*.

Particularly because of the cycle of development of these two species, Imseneckı and Solntzeva (1936) considered all the cytophagas, both microcystogenous and amicrocystogenous, to be reduced myxococci. They suggested that *S. myxococcoides* and *S. ellipsospora* were *Myxococcus* species which had lost completely the ability to form organized fruits or which failed to do so under laboratory conditions. The remaining types they regarded as still further reduced or amicrocystogenous races of the already aberrant *Sporocytophaga* species. It was postulated that *C. hutchinsoni* was a race of *S. myxococcoides* and *C. aurantiaca* of *S. ellipsospora*. The substantial uniformity of nutrient requirements in the group was undoubtedly an important contributing factor in the development of these ideas.

The discovery of the amicrocystogenous marine cytophagas drastically revised our concepts of nutritional requirements among these organisms, and showed that the group was a broader one in this respect than had been previously realized. However, even considering the soil species alone, there are a number of facts which indicate that the ideas of Imseneckı and Solntzeva were somewhat oversimplified.

Is it possible that in nature the *Sporocytophaga* species would form typical myxococcus fruits? This seems most unlikely. It must be remembered that the loss of ability to fruit normally under laboratory conditions, which has often been noted among the higher myxobacteria, is due first and foremost to cultivation on unsuitable media. Methods for isolation of the fruiting myxobacteria have been purely empirical and have allowed no insight into their natural activities and biochemical peculiarities. Because these organisms occur naturally on dung, the assumption developed that they were primarily coprophilic. As a result, dung decoctions of various types have been commonly used for laboratory culture, even though in many cases it was evident that the requirements for normal fruiting were not being fulfilled by such substrates. The recent work of Snieszko, *et al* (1941, 1942), of Beebe (1941) and of myself (unpublished) has shown that many of the higher myxobacteria are active and specialized destroyers of other forms of microbial life. When grown with yeasts or bacteria as a food source, species which fruit poorly or not at all on dung agar will fruit normally and abundantly. It is now clear that their frequent occurrence on dung is due largely to the exceptionally high bacterial content of this material.

*Sporocytophaga* species develop in a strictly defined enrichment medium, often to the practical exclusion of other cellulose decomposers. It is a microbiological axiom that the enrichment culture technique permits an insight into the natural

activities, the micro-ecology, of microbial groups. In this case the biochemical specialization of the *Sporocytophaga* species as determined by means of pure culture studies provides convincing support for the deductions which can be drawn from enrichment experiments, and makes it certain that under the competitive conditions of a natural environment these organisms can exist solely through attacking cellulose, with inorganic compounds as their usual nitrogen source. Hence by cultivating them in a mineral medium with cellulose as the sole source of carbon, we may be certain that we are closely approximating the conditions of their natural activities. Yet not even on first enrichment plates, where the organisms are still for the most part in contact with soil particles, is there ever any sign of true fruiting-body formation. Thus the suggestion that *S. myxococcoides* and *S. ellipsospora* are typical myxococci which do not fruit normally under laboratory conditions seems unjustified.

We must now consider Imseneck's and Solntzeva's concept of the *Cytophaga* species as amicrocystogenous sporocytophagas. Although at first sight it might seem reasonable to assume that this is true of *C. hutchinsonii* and *C. aurantiaca*, we do not know of the existence of microcystogenous equivalents for the other soil and for the marine species, which must then be regarded as independent forms. Furthermore, the permanent loss of the ability to form microcysts has not been experimentally demonstrated, although if this is a common occurrence in nature, as Imseneck and Solntzeva seem to imply, it should be easily brought about under laboratory conditions. Even were it possible to produce an amicrocystogenous race of *S. myxococcoides*, my work has shown that it could be distinguished from *C. hutchinsonii* in a number of ways. Without denying the likelihood that a close relationship exists between the soil *Cytophaga* and *Sporocytophaga* species, I feel that the available evidence supports the contention that they are independent assemblages.

Although it is thus fairly certain that the *Sporocytophaga* and *Cytophaga* species are not merely variant forms of true myxococci, it is more difficult to decide whether from the evolutionary standpoint they should be regarded as derived from fruiting members of the *Myxococcaceae* or as primitive forms. The discovery of new representatives may throw some light on this, but a final answer to such questions is very rarely possible. In the ultimate analysis, pronouncements on phylogenetic problems are guesses based on the accumulated knowledge and scientific tact of the investigator concerned.

There are two ways in which the cytophagas may be incorporated into the *Myxobacteriae* as this class has been defined above.

The first possibility is to combine the genera *Sporocytophaga* and *Cytophaga* into a single family, or perhaps order, which would be set apart from the remaining groups as containing non-fruiting myxobacteria, a sort of *Myxobacteria Imperfecta*. The second possibility is to include the genus *Sporocytophaga* with the family *Myxococcaceae*, and create a new family for the amicrocystogenous group alone. There are advantages and disadvantages to both approaches.

In the former case, the obvious relationships between the *Myxococcaceae* and *Sporocytophaga* are not brought out, and the important character of microcyst

formation assumes a minor position. On the other hand, the two groups of cytophagas, which are also related to each other, remain together, and the differences in structural complexity between them and the higher myxobacteria receive full expression.

In the latter case, the inclusion of the genus *Sporocytophaga* in the *Myxococcaceae* preserves expression of the *Sporocytophaga-Myxococcus* relationship and retains the property of microcyst formation as the major differential character of the *Myxococcaceae*. However, the two groups of cytophagas become more widely separated than is desirable, and the ability to form fruits assumes a position of minor taxonomic importance.

Which solution to adopt is a difficult problem, and can necessarily only be settled in an arbitrary way. It depends entirely on whether one wishes to regard the formation of organized fruits or the formation of microcysts as the more important character. I have decided to use the latter policy by placing the genus *Sporocytophaga* in the *Myxococcaceae* and creating a new family *Cytophagaceae* for the amicrocystogenous species (Stanier, 1940). There are indications from the work of Imsenecki and Solntzeva that in *S. ellipsospora* a sort of primitive fruiting occurs by the formation of small microcyst aggregates, a finding which tends to show that the separation of fruiting and non-fruiting myxobacteria may not always be a simple matter. On the other hand, the separation of microcystogenous and amicrocystogenous forms is perfectly clear cut, so that from the determinative standpoint, at least, this system of separation is perhaps the most satisfactory one. Of course, the possibility remains that amicrocystogenous races will be derived experimentally from *Sporocytophaga* species and will have to be placed in a separate family from the parent organism. The same type of difficulty exists, however, in the *Eubacteriae*, where the family *Bacillaceae* is defined solely on the property of endospore formation even though asporogenous races of *Bacillus* species are known.

The following key to the families of the *Myxobacteriae* (there is only one order, *Myxobacteriales*, with definition as for class) will make clear the proposed arrangement.

- I Vegetative cells thick and short, with blunt, rounded ends
  - A Microcysts rod-shaped
 

Family I *Sorangiaceae*
- II Vegetative cells long and thin, sometimes spindle-shaped with pointed ends
  - A Microcysts rod-shaped
 

Family II *Polyangriaceae*
  - B Microcysts spherical or oval
 

Family III *Myxococcaceae*
  - C Microcysts absent
 

Family IV *Cytophagaceae*

The definition of the family *Myxococcaceae* must be amended to read "The long slender rods become shortened to form spherical or oval microcysts, definite fruiting bodies may or may not be formed."

The family *Cytophagaceae* (Stanier, 1940) is defined as follows "Long slender rods. Neither fruiting bodies nor microcysts formed."

*Detailed taxonomy and descriptions of species.*

The inclusion of the genus *Sporocytophaga* in the *Myxococcaceae* necessitates a revision of the genera in this family Jahn's (1924) treatment, as accepted by Buchanan in the fifth edition of Bergey's Manual, left something to be desired in any case His key to the genera runs as follows

- I Spores lie in a loose slime Fruiting body spherical or lengthened to columnar At the base there may develop a kind of foot as a result of abundant slime formation  
I *Myxococcus*
- II Spores united by a viscous slime into fruiting bodies of a definite form
  - A Fruiting body constricted or branched like coral  
II *Chondrococcus*
  - B Spores lie in cysts of definite shape  
III *Angiococcus*

The following amended key, including the genus *Sporocytophaga*, is proposed to take its place

- I No cysts formed
  - A Definite fruiting bodies formed
    - 1 Fruiting bodies deliquescent  
I *Myxococcus*
    - 2 Fruiting bodies not deliquescent  
II *Chondrococcus*
  - B No definite fruiting bodies formed  
III *Sporocytophaga*
- II Cysts formed  
IV *Angiococcus*

Such a separation has several advantages over Jahn's Although intermediate forms may later be found, at present the sharpest division in the *Myxococcaceae* is one based on the presence or absence of cysts On the other hand, the dividing line between *Myxococcus* and *Chondrococcus* is not a sharp one The position of a number of species depends entirely on whether one uses the shape of the fruiting body or its deliquescence as a criterion Jahn tried to use both, with the result that neither was clearly stressed Examples of intermediate forms impossible to place unless the distinction between these two genera is made more rigorous, are *Myxococcus cruentus* Thaxter and *Myxococcus exiguus* Kofler The former species was restudied by the Krzemieniewskis (1930), they found that although its fruits were very similar in color, size and shape to those of *M. fulvus*, they were non-deliquescent and surrounded by a firm membrane Consequently the Krzemieniewskis placed this species in the genus *Chondrococcus* My studies on *M. exiguus* (unpublished) have revealed a similar situation

The statement by Jahn that the fruiting bodies in the genera *Angiococcus* and *Chondrococcus* have a "bestimmte Form", with the implication that this is not the case for the genus *Myxococcus*, is also misleading The fruits of *M. fulvus* and *M. virescens* certainly have a definite form, whereas those of *C. coralloides* are highly variable in size and shape

The genus *Sporocytophaga* is defined as follows

Spherical or ellipsoidal microcysts formed loosely among the vegetative cells  
 Fruiting bodies absent

The type species is *Sporocytophaga myxococcoides* (Hutchinson and Clayton emend Krzemieniewska) Stanier. At present only one other species, *Sporocytophaga ellipsospora* (Imseneckı and Solntzeva) emend, can be recognized with certainty as belonging to the group. *S. ellipsospora* is sharply distinguished from *S. myxococcoides* by its oval microcysts and orange color. *C. globulosa* Stapp and Bortels differs in no essential respect from *S. myxococcoides* and must be considered a synonym. A species more difficult to place is the one described by Rippel and Flehmig (1933) under the name *Itersonia ferruginea*. In many ways this organism bears a suspicious resemblance to *S. myxococcoides*, although its vegetative morphology appears to be somewhat different. Rippel and Flehmig themselves considered the two organisms to be distinct, but the reasons for this are not clearly given. It is likely that some of Johnson's (1932) chitin-decomposing myxobacteria belong to the genus *Sporocytophaga*. The descriptions given were far from complete, and as Johnson did not name any species they are perhaps best left out of consideration until more detailed studies have been made.

The following descriptions are based on my own work unless otherwise stated

Key to the species of the genus *Sporocytophaga*

I Microcysts spherical

1 *Sporocytophaga myxococcoides*

II Microcysts oval

2 *Sporocytophaga ellipsospora*

1 *Sporocytophaga myxococcoides* (Hutchinson and Clayton emend Krzemieniewska) Stanier (1940). Synonyms: *Micrococcus cytophagus* Merker (1912), *Spirochaeta cytophaga* Hutchinson and Clayton (1919), *Cytophaga myxococcoides* Krzemieniewska (1933), *Cytophaga globulosa* Stapp and Bortels (1934), *Cytophaga hutchinsonii* Imseneckı and Solntzeva (1936), *Myxococcus hutchinsonii* Imseneckı (1940) not *Cytophaga hutchinsonii* Winogradsky (1929).

Vegetative morphology: Flexible, weakly refractile, singly occurring rods, 0.3-0.4 by 2.5-8.0 microns. Gram negative. The young cells stain uniformly with Winogradsky's or Giemsa's stain. As the rods begin to shorten and round up prior to microcyst formation, the chromatin material becomes concentrated in bands or spots which are usually centrally located. Star formation is common in liquid media.

Microcysts: Spherical, varying in size from 1.2 to 1.6 microns. The microcysts are surrounded by a highly refractile wall or sheath. They stain evenly and intensely with many stains.

Growth on cellulose: On mineral silica-gel plates or agar plates covered with a piece of filter paper, glistening, light yellow patches become apparent after 4-5 days. These patches spread slowly, while the central areas become translucent due to the complete lysis of the cellulose fibers. Old cultures often assume a brownish tinge.

In tubes of liquid mineral medium with filter paper partly immersed, growth occurs as a pale yellow region at the air-water interface. There is never any visible development below the water level, but so long as the upper part of the strip remains moist the growth will spread on the paper above the liquid surface. The liquid itself remains perfectly clear. After 5-7 days the filter paper at water level has become completely dissolved, in its place there is a membranous yellow "skin" composed of organisms held together by slime.

Growth on filtered glucose: In tubes of mineral medium with filtered glucose growth is granular and uneven, with very little turbidity. On the glass walls and in the liquid there occur small yellow floccules.

On mineral filtered glucose agar plates, two colony types appear after 3-4 days. One type consists of very small, round colonies with even edges. The other type is flat and irregular with an uneven edge where active swarming takes place. Both are pale yellow and translucent. As the culture ages, the former type breaks down and begins to swarm, so that the differences between the two types tend to disappear. The agar beneath the colonies becomes irregularly etched and sunken, although actual agar decomposition apparently does not take place.

Utilizable carbon sources: Cellulose, cellobiose and glucose

Utilizable nitrogen sources:  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{KNO}_3$ , aspartate, asparagin, peptone, yeast extract, urea

Catalase positive

Strictly aerobic

Temperature range: 20-35°C Optimum 35°C

Source and habitat: Soil Extremely common

2 *Sporocytophaga ellipsospora* (Imseneck and Solntzeva) *nov comb* Synonym *Cytophaga ellipsospora* Imseneck and Solntzeva (1936)

Description taken from Imseneck and Solntzeva

Vegetative morphology: Flexible, singly occurring cells. Average dimensions 0.45 by 7.5 microns

Microcysts: Oval or somewhat elongated, 0.9 by 1.65-1.8 microns. Not as refractile as the microcysts of *S. myzococcoides*. Usually occur in aggregates

Growth on cellulose: On mineral silica-gel plates covered with filter paper there appear glistening, mucoid, orange spots. As the orange areas spread, the central portions become translucent owing to complete lysis of the fibers.

In tubes of liquid mineral medium with filter paper partly immersed, an orange strip or separated orange spots are found at the air-water interface or a little above it. The colored area gradually spreads upwards on the filter paper, and in its place there develops a thin, transparent pellicle similar to that formed by *S. myzococcoides*.

Utilizable carbon sources: Only known one is cellulose. Others have not been properly tested.

Utilizable nitrogen sources: Ammonia, nitrates, peptone. Others have not been tested.

Strictly aerobic (Jensen, 1940)

Temperature range: 15-37°C Optimum 28°C

Source and habitat: Soil

In the family *Cytophagaceae*, there is only one genus, *Cytophaga* Winogradsky emend., with description as for family. The genus is thus now defined entirely on morphological characters, without any of the reservations as to nutrient requirements which were proposed by Winogradsky. Such a treatment, desirable on general grounds alone, has been made necessary by the discovery of the marine cytophagas and the revision of our ideas on the nutrition of the soil cytophagas.

The number of valid species is a debatable point. The two marine agar-decomposers are clearly distinct from each other and from the other members of the genus, but among the soil forms matters are less clear-cut. Winogradsky's four species *C. hutchinsonii*, *C. rubra*, *C. aurantiaca* and *C. tenuissima* may be differentiated on the basis of pigmentation, although only for the first two species do we have adequate descriptions. Winogradsky's *C. lutea*, which differed from *C. hutchinsonii* in size and shape of the vegetative cells alone, to judge from his descriptions, has a somewhat doubtful status in view of the variability of these characters. Hence this species should perhaps be regarded as synonymous with

*C. hutchinsonii*, at least until some further methods of distinguishing between them have been found. The same applies to the four species described by Stapp and Bortels (1934). We are thus left with six species in the genus, of which *C. hutchinsonii* is the type.

Key to the species of the genus *Cytophaga*

- I Soil group. Specialized cellulose decomposers, with a limited range of carbon sources. Cannot develop in the absence of carbohydrates. Grow well in a mineral medium with a suitable carbohydrate and an inorganic nitrogen source. Optimum temperature 30°C.
  - A Yellow pigment produced
    - 1 *Cytophaga hutchinsonii*
  - B Orange pigment produced
    - 2 *Cytophaga aurantiaca*
  - C Pink pigment produced
    - 3 *Cytophaga rubra*
  - D Olive green pigment produced
    - 4 *Cytophaga tenuissima*
- II Marine group. Not specialized cellulose-decomposers, can utilize a wide range of carbon sources. Can develop in the absence of carbohydrates. Cannot grow with inorganic nitrogen sources alone. Optimum temperature 22–25°C.
  - A Produces a black diffusible pigment
    - 5 *Cytophaga krzemienievskae*
  - B Does not produce a black diffusible pigment
    - 6 *Cytophaga diffluens*

1 *Cytophaga hutchinsonii* Winogradsky (1929). Synonyms *Cytophaga lutea* Winogradsky (1929), *Cytophaga sylvestris*, *Cytophaga anularis*, *Cytophaga flavicula* and *Cytophaga crocea* Stapp and Bortels (1934), not *Cytophaga hutchinsonii* Imseneck and Solntzeva (1936).

**Vegetative morphology.** Flexible, weakly refractile, singly occurring rods, 0.3–0.5 by 2.0–10.0 microns. Length very variable, average about 6.0 microns. Gram negative. Stain uniformly with Winogradsky's or Giemsa's stains. Thread-like or coccoid involution forms may occur in old cultures.

**Growth on cellulose.** On mineral silica gel or agar plates covered with filter paper, bright yellow, glistening, highly mucilaginous patches appear after 3–5 days and spread rapidly. Fibrolysis is intense, and the older portions of the colonies soon become transparent. Old cultures retain their bright yellow color almost indefinitely.

In tubes of liquid mineral medium with filter paper partly immersed, growth becomes apparent after 3–5 days as a bright yellow area on the paper at the air-water interface. The growth area has a tendency to spread upwards, but there is never visible development below water level. The liquid rapidly becomes turbid and semi-opaque, this is not caused by the organisms themselves, but is due to the gum which they produce, and which partly dissolves to give an opalescent solution. After about five days the paper at the water level is completely decomposed and tends to break. A pellicle of cells and firm slime is never substituted for the paper as in *S. myxococcoides*.

**Growth on filter-sterilized glucose.** In liquid cultures growth is at first even throughout the liquid and characteristically "silky". Turbidity is very marked, but this is largely due to gum production. In cultures with more than 0.5 per cent glucose a heavy, slimy, yellow ring or pellicle often forms on the surface.

On mineral glucose-agar plates, bright yellow, glistening, slimy raised colonies develop. In young cultures grown at 30°C, swarming masses can be observed moving out here and there from the periphery, but in older cultures and on plates grown at room temperature swarming is almost completely absent. There is never any etching of the agar.

Utilizable carbon sources Cellulose, cellobiose and glucose  
 Utilizable nitrogen sources Ammonia, nitrates, aspartic acid, asparagin (some strains),  
 peptone, yeast extract  
 Catalase positive  
 Strictly aerobic  
 Temperature range 15-30 C Optimum 30 C  
 Source and habitat Soil Extremely common

2 *Cytophaga aurantiaca* Winogradsky (1929) Synonym *Mycococcus cytophagus* Bokor (1930) *pro parte* (?)

Description taken from Winogradsky  
 Vegetative morphology Flexible, singly occurring rods, 1.0 by 6.0-8.0 microns Very similar to *C. hutchinsonii*  
 Produces orange mucilaginous patches on mineral silica gel plates with filter paper  
 Fibrolysis is very rapid and intense  
 Source and habitat Soil

3 *Cytophaga rubra* Winogradsky (1929)

Vegetative morphology Flexible, weakly refractile, singly-occurring rods, 0.5-0.7 by 3.5-11.0 microns Very variable in length, average about 7.0 microns Gram negative Cells stain evenly with Winogradsky's or Giemsa's stain

Growth on cellulose On mineral silica gel or agar plates covered with filter paper, diffuse, bright pink, rapidly spreading patches appear after a few days The colored patches are only very slightly mucilaginous Fibrolysis is much less marked than with *C. hutchinsonii*, the action on cellulose is invasive rather than destructive

In tubes of liquid mineral medium with filter paper, bright pink growth becomes apparent after 2-3 days While strongest at the air-water interface, it also occurs above and (in marked contrast to the other species) below the water level Many cells occur free in the medium, which becomes faintly turbid and silky, with a pale pink color The attacked filter paper becomes weak and macerated, but there is never a complete lysis On shaking, the attacked paper breaks up into particles and shreds of fibers

Growth on filter-sterilized glucose In liquid cultures an even turbidity is produced The growth is pale pink and silky

On glucose mineral agar plates, colonies are always very small and inconspicuous, never attaining a size of more than 2 mm They are pale pink, translucent and sunken in the agar, with hazily defined peripheries Swarming is never apparent

Utilizable carbon sources Cellulose, cellobiose, glucose, mannose and xylose

Utilizable nitrogen sources Ammonia, nitrates, aspartic acid, asparagin, peptone and yeast extract

Catalase positive

Strictly aerobic

Temperature range 15-30 C Optimum 30 C

Source and habitat Soil Fairly common

4 *Cytophaga tenuissima* Winogradsky (1929)

Description taken from Winogradsky

Dimensions of cells not given, but described as being extremely tenuous

Produces mucilaginous olive-green patches on filter paper silica gel plates

Source and habitat Soil Probably rare

5 *Cytophaga krzemieniewskae* Stanier (1940)

Morphology Long, very flexible rods, 0.5-1.5 by 5.0-20.0 microns Gram negative Star-shaped aggregates are common in liquid media Coccoid involution forms frequently occur in old cultures

Sea-water peptone agar plate Growth begins as a smooth, pale pink, rapidly spreading swarm After a few days, the older portion of the swarm assumes a warty appearance due

to the accumulation of the cells in drop-like masses resembling immature fruiting bodies, but always containing normal vegetative cells. A diffusible brown to black pigment which masks the pink color of the swarm is produced after about a week. Agar is rapidly decomposed, and ultimately liquefaction becomes almost complete.

Sea-water gelatin stab Liquefaction

Growth in liquid media (sea-water peptone broth, sea-water glucose peptone broth) is turbid and silky with a pink sediment, the medium turns dark brown or black after 1-2 weeks.

Utilizable carbon sources Xylose, glucose, galactose, lactose, maltose, cellobiose, cellulose, alginic acid, agar and starch, also proteinaceous materials

Utilizable nitrogen sources Yeast extract and peptone are the only suitable ones known

Weakly catalase positive

Indole not formed

Salt concentration range 1-5-0 per cent

Strictly aerobic

Temperature range 15-30 C Optimum 22-25 C

Source Sea water

Habitat Probably decaying marine vegetation

#### 6 *Cytophaga diffluens* Stanier (1940)

Morphology Flexible rods, 0.5-1.5 by 4-10.0 microns. Gram negative. In old cultures involution forms consisting of long, twisted, thin threads are found. Star-shaped aggregates of cells occur in liquid media.

Sea water peptone agar plate Growth begins as a thin, pink, rapidly spreading swarm which covers the entire surface in a few days. The swarm gradually increases in thickness and develops an irregular, beaten-copper surface due to the liquefaction of the underlying agar. After 4-5 days the color becomes orange. Liquefaction of the agar is ultimately almost complete.

Sea-water gelatin stab Rapid liquefaction

Growth in liquid media is turbid, often with suspended floccules and a heavy pellicle.

Utilizable carbon sources Xylose, glucose, galactose, lactose, maltose, cellobiose, cellulose, agar and alginic acid, also proteinaceous materials

Utilizable nitrogen sources Yeast extract and peptone are the only suitable ones known

Weakly catalase positive

Indole not formed

Salt concentration range 1-5-0 per cent

Strictly aerobic

Temperature range 15-30 C Optimum 22-25 C

Source Sea water

Habitat Probably decaying marine vegetation

#### ADDENDUM

After this monograph had been completed, there appeared a short note by Fuller and Norman (J. Bact., 44, 256) in which several new soil forms were described. These organisms differ from the classical soil cytophagas through their far greater physiological versatility, growing vigorously on a wide variety of simple carbohydrates and even, in some cases, not attacking filter paper. Through the kindness of Dr. Norman I have been able to examine several of these cultures. They conform to the requirements for inclusion in the cytophaga group as presented above, and thus furnish an interesting biochemical parallel to the "unspecialized" marine species. Further work may well show that the non-fruiting myxobacteria comprise a large and varied assemblage.

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# TISSUE PERMEABILITY AND THE SPREADING FACTORS IN INFECTION<sup>1</sup>

## A CONTRIBUTION TO THE HOST PARASITE PROBLEM

F DURAN-REYNALS

*Department of Bacteriology, Yale University School of Medicine, New Haven, Connecticut*

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The extraneous cell coatings and intercellular cements present throughout the zoological scale have recently been given deserved attention (28, 29, 123, 98, 45) (See 152, 7, 257 for theories on the origin and nature of one of these substances, the ground substance of the mesenchyme, in embryonal life) In the present review we shall be concerned mainly with the ground substance, in the post-embryonal stage of higher vertebrates, in which it occurs in all varieties of simple and specialized connective tissues, and in other locations Since most substances reaching cells must pass through the ground substance, infectious agents and their secretions must also traverse it In this ground substance the phenomena of inflammation occur and therefore analysis of its nature is important to bacteriologist and physiologist alike Infection is too often described as developing between cells, humoral bodies, and infectious agents without much regard for the field where it takes place Efforts by bacteriologists to clarify the importance of this field in infection, and the results of direct experiments by a few physiologists, have provided facts which form a basis for the concept of *permeability* of the connective tissue (C T) It would seem that all these facts could be properly ranged with or between those concerning *cell permeability* and *capillary permeability* It will be shown that the permeability of the C T has a normal, constitutional state or tonus which can be increased by certain factors and decreased by others, and these fluctuations of permeability result in parallel fluctuations in the degree of the infection which may have a decisive bearing on the final issue of the process Of the factors increasing the permeability of the C T undoubtedly the most important are the spreading factors (S Fs), some of which are today identified with mucolytic enzymes To these factors a great part of our study will be devoted

<sup>1</sup> Practically all of the work by the author on the subject was carried out in the Rockefeller Institute of New York in the Department of Dr J B Murphy to whom we are thankful for the facilities given

## SOME MORPHOLOGICAL AND FUNCTIONAL DATA ON THE CONNECTIVE TISSUE

*Origin and distribution of the ground substance* It is generally accepted that, in adult life, the ground substance is a product of secretion of the C T cells (152, 257, 101, 11) and that through progressive transformations of this substance, argyrophilic fibers, collagen, and the matrices of all mesenchymal tissues are formed (101, 152) Its distribution has been studied by S Bensley (11), who routinely used toluidine blue to detect it She showed that in the guinea pig pancreas after duct ligation, and in the human uterine mucosa, there is a succession of phases edema  $\rightarrow$  gelatinous ground substance  $\rightarrow$  argyrophilic fibers  $\rightarrow$  collagen, the ground substance being considerably reduced at the last phase The distribution of the ground substance was found to be the same in the umbilical cord, intima and media of blood vessels, C T of lower vertebrates, gastric mucous membranes, and in general in all reticular and embryonic tissues

*Physical and chemical properties* Bensley's work has disclosed some general properties of the ground substance By mounting fresh tissues in a medium of a different refractive index it is seen as a continuous, fairly granular, transparent substance in which fibers are imbedded It is elastic and tends to retract when cut As judged by the reactions of paramedica injected into the C T, it is viscid and possibly acid Its refractive index is between 1.33 and 1.34 It is extractable with 10% salt solution, and half-saturated lime water It is digested by trypsin but not pepsin in contrast to collagen where the reverse is true It combines with copper salts suggesting that it may contain a derivative of chondroitin sulphuric acid

The same year that Bensley reported her histological observations Meyer, *et al* contributed the first chemical observations on compounds whose identity with those present in the ground substance was later to be established These compounds are of basic interest to us because they are the substrates of many S F's In 1934 Meyer and Palmer (183) reported the occurrence in vitreous humor of cattle eyes of a polysaccharide consisting of equimolecular parts of hexosamine (d-glucosamine), hexuronic (glucuronic) acid, and acetyl, the latter apparently as N-acetylglucosamine They named this compound hyaluronic acid Later, they duplicated the finding on aqueous humor and Wharton's jelly (184, 186) and on synovial fluid from cattle (185) Kabat (120) has isolated hyaluronic acid from a leucotic tumor of the fowl, and Meyer and Chaffee (175, 177a) have obtained it from a mesoendothelioma of the human pleura and peritoneum Several authors have extracted from rabbit skin (27, 41) and from rabbit fasciae (214) a mucoid substance showing typical reactions of hyaluronic acid, and the acid has been isolated from pig skin (178) A monosulfuric acid ester of hyaluronic acid has been isolated from cattle cornea (177) Hyaluronic acid is absent from blood, cerebrospinal fluid, and nasal and gastric mucin It has been found but in varying concentrations in the ocular humor of 7 animal species including man, being highest in cattle It has a high molecular weight, and does not dialyze through collodion membranes of great porosity It is viscid, soluble in water, and precipitable by acetic acid It occurs free or in salt linkage, and is not chemically bound to protein, but it will combine stoichiometrically with the free amino groups of proteins at pH's lower than the iso-

electric point of the proteins to form salts. The so-called mucins or mucoids prepared by acidifying the diluted solutions of such fluids as synovial fluid or vitreous humor are such salts. They are artefacts, which apparently do not occur in nature. In all fluids so far investigated hyaluronic acid migrates in the electric field at pH 7.6–7.8 with the same speed as the isolated pure acid. Hyaluronic acid is not antigenic (173a, 177a). The same compound, also serologically inactive, has been isolated (121) from mucoid strains of streptococcus.

*Staining properties.* There is great need for a specific microchemical test for compounds of hyaluronic acid to detect its exact distribution in tissues whose intercellular cements are still imperfectly known (29, 253, 30). The ground substance had been described as staining with various dyes, but none of the usual methods of coloration of the C.T. is specific. Toluidine blue and thionin have been much used to distinguish between mucin (staining violet) and nuclei (blue).

Bensley (11) concluded that toluidine blue, although not a microchemical test for the ground substance stains it differentially and metachromatically, an effect shared to a certain extent by thionin. Sylven (239) proved the selectivity and superiority of toluidine blue as compared to mucicarmine, and showed that "mesodermal mucus" is the result of C.T. activity and not one of degeneration. It was known that toluidine blue stained blue most elements of a histological section, but several structures stained metachromatically in purple or red. Lison (130) reported that all esters of sulfuric acid of high molecular weight including such polysaccharides as mucicarmine and chondroitin sulfuric acids and heparin, substances closely allied to hyaluronic acid, gave the metachromatic reaction which he considered as specific. However, Kelly and Miller (120a) have found the reaction to take place with some isolated components of the nuclei, and in all probability the reaction is positive with all substances of high molecular weight having an acidic function (173a) (See 120a for further data).

Heparin has been identified by Jorpes as a mucicarmine polysulfuric acid, giving a typical metachromatic reaction. Subsequently, there was demonstrated (116, 117, 239) the identity between heparin and the metachromatic granules of mast cells. But besides these cells, several other structures (capsule of some organs, vessel walls, eye tissues, stroma of neoplasms, etc.), and in close association with elastic fibers, smooth muscle and endothelium) were found to contain large amounts of extracellular metachromatic substance. Although the occurrence of this substance in tissues and mast cells was always proportional to their content of esters of sulfuric acid of polysaccharides, lack of correlation between the amount of extracellular metachromatic substance and that of mast cells occurred frequently. In such cases, it has been assumed that the extracellular substance was not heparin but ordinary chondro- and mucicarmine-sulfuric acids. (See review by Best (12) for additional data.)

The only true derivative of hyaluronic acid found in the body thus far is the hyaluronic sulfuric acid ester of the cornea (177), a tissue giving a beautiful metachromatic stain (117). It has first thought that hyaluronosulfuric acid likewise occurred in pigskin, subsequently, however, it was found (178) that the first preparations were mixtures which could be separated into free hyaluronic acid and a second sulfate containing acid polysaccharide believed to be an isomer of chondroitin sulfuric acid.

Therefore, the general occurrence of sulphur-containing polysaccharides other than hyaluronic acid (see 174) makes it difficult to evaluate to what extent compounds of hyaluronic acid are responsible for the metachromatic stain in the skin as well as in other tissues. Also, the significance of the reaction in struc-

tures such as the Nissl granules and some components of unicellular organisms remains to be determined. Staining with toluidine blue after treatment of tissue slices with hyaluronidase might be a means of improving the method.

*Data from studies on articular structures and neoplasms* These data are helpful in interpreting phenomena of infection or other processes taking place in the C T under comparable conditions. It is interesting to consider that there is many a common point in the theories proposed to explain the formation of the synovial and ocular fluids, and the formation of the ground substance of the C T (122, 250, 9). The case of the synovial membranes is particularly illuminating since it is now generally accepted that the synovial membrane is modified C T.

As far back as 1886, Retterer regarded the primitive tissue between embryonic cartilages as the result of the fusion of clefts, where mucin accumulates, appearing in the mesenchymatous syncytium (see 250), and Vaubel (250), after his work on *in vitro* culture of synovial cells, extended this conception to adult life. "The histological and physiological properties of the synovialis, its great reparative and proliferative power and the nature of its cytological response to injury all indicate that the articular lumen represents a connective tissue space" (9). The recent work by Meyer, *et al* has added to this by showing that hyaluronic acid is a common component of synovial fluid and of the ground substance. A type of synovial cell, the "synovioblast" of Vaubel has been found to contain in its cytoplasm granules which stain bluish or reddish with toluidine blue while others remain unstained. Such cells secrete mucin as well as proteolytic enzymes, both being evident in tissue culture (250). Mucin production is not the result of cell death but of cell activity, and, in inflammation, the number of cytoplasmic granules and the secretion of mucin increases greatly (122). When synovioblasts change into fibroblasts there is a diminution of mucin secretion. Toluidine blue stains the ground substance of the synovial membranes metachromatically, and this substance also blackens upon silver impregnation.

Neoplasia exaggerates the production of hyaluronic acid by tissues which secrete it normally. Such is the case in the primary and metastatic neoplasias of the synovial tissues, which has been shown (122) to secrete large amounts of synovial mucin, and in the highly viscid sarcomata of birds (120). But in other cases, *e g*, the endotheliomas of pleura and peritoneum (177a), the presence of the acid in the normal tissue from which the neoplasia has arisen has not been reported.

*Data on blood and lymph vessels* Studies by several histologists, some of them dating back to 1851, strongly suggested the existence, in some tissues, of an adventitia in blood capillaries (see 253). Volterra (253, 252) has conclusively shown that the adventitia represents an indispensable and constant element of all capillaries. Silver impregnation shows it to consist of a delicate reticulum spread on and merged with an amorphous fundamental substance, and the whole structure forms a complete membrane composed of what Volterra calls lamellated reticular tissue which is continuous with the fibers of the surrounding C T. Recent studies (35, 36, 115, 206) have added further to the intimacy of the relations between C T and blood and lymph capillaries. The studies of Chambers (30) and of Zweifach (260) have defined the cement present between the endothelial cells of blood capillaries as a sticky, argyrophilic matter, continuously secreted by the cells themselves, and conditioned by Ca salts and the pH level. The cement behaves as if it were a salt of Ca, is digested by trypsin, and is not laid down in the absence of vitamin C.

*The motion of matter through the C T Tissue permeability* Despite the existence of many varieties of C T one can visualize the field, where the phenomena to be described take place, as consisting essentially of a viscid ground substance in which argyrophilic fibers, collagenous bundles, cells and fibroglia, and lymph and blood vessels are embedded. One is struck by the resistance offered by the C T to penetration by foreign fluids introduced either by micromethods or by simple intradermal injections, and the interstitial dispersion of materials toward cells must be achieved by overcoming a strong barrier.

There was current a belief that between cells and fibers of the C T channels existed, and that through these "spaces" free fluid moved. However, the Clarks (35), by using the transparent chamber method, observed that there is no Brownian movement in the jelly-like ground substance, and concluded that normally these are no tissue spaces in the strict sense. McMaster and Parsons (169) approached the problem by studying the escape of vital dyes injected into lymphatic capillaries in a variety of C Ts of the mouse and rabbit. In the absence of edema, the dye escapes from the vessels as bristly, wavy lines of color formed by the dye moving between or along the C T fibrils. The fluid moves through tissues in thin films "captured" by capillary forces as if caught between two pieces of glass (169). It never oozed from a microscopic stab wound in normal skin (199). Wolbach (257) had already found evidence that fibroglia cells and fibers are conductors of the agents determining the alignment and distribution of collagen. In subsequent studies, McMaster (167) showed that the resistance of the C T to penetration by fluid is not overcome until a pressure of 8.5 cm of water has been reached.

In summary, despite the viscous consistency of the ground substance, metabolites must move easily through it, probably impelled by capillary forces. But as a result of this viscous consistency, the ground substance offers a pronounced resistance to penetration by foreign matter, among which infectious agents and their secretions must naturally be included. Variations in this resistance are the only or the main determinants of the permeability of the tissue.

#### PHYSIOLOGICAL FACTORS MODIFYING THE PERMEABILITY OF THE CONNECTIVE TISSUE AND THEIR INFLUENCE ON INFECTION AND RESISTANCE

Early in the work on S Fs it was observed that although physiological factors were the most powerful in modifying the permeability of the C T they were not the only ones. For example, the age and race of the host, and individual and regional characteristics of the skin were found to be important determinants (57). Judging by observations on the skin there is in each individual a normal state of permeability which does not change fortuitously, but rather obeys rules. Before reviewing some of the factors conditioning tissue permeability, some data concerning the technics employed should be set forth.

*Methods of measuring the permeability of the C T* These consist of interstitial injections, generally into the skin, of a colored matter, *i e*, diluted India ink, poorly diffusible dyes such as T 1824 (Evans blue), Pontamine sky blue, hemoglobin (140), or some toxic substance such as diluted diphtheria toxin (153) which will induce a slight local reaction. The extent of a local infection is in itself an indicator of permeability. To study the possible effect of a substance on tissue permeability, the material is mixed with a colored indicator and the mixture is injected intradermally generally in rabbits. A corresponding area of the skin is

injected with the indicator mixed with saline solution. When using India ink a certain amount of inactivation of some spreading materials by adsorption to the carbon particles takes place (87, 241). The areas of the blebs or spots resulting from the injections and the density of the discoloration must be observed immediately and at different intervals after injection. They are generally recorded after 24 hours. Sometimes it is useful to make injections in duplicate: the first allows study of the spontaneous undisturbed spreading, and the second makes possible observation of the forced spreading which results when pressure is exerted on the inoculation bleb immediately after injection. The method of wheal disappearance time proposed by McClure and Aldrich (166) can in several respects be considered as measuring the tissue permeability (235). (See publications of McMaster for microinjection techniques.)

*Constitutional Factors* The most important studies are those carried out in relation to tuberculosis by Lurie (135) and Lurie and Zappasodi (136) on six families of inbred rabbits. When individuals of family A, the most resistant, were exposed to infection by natural means they developed a local ulcerative disease of reinfection type comparable to human phthisis. When family F, the most susceptible, was similarly infected, a widespread disease with the characteristics of a first infection and resembling the childhood type in man developed. When the skin permeability of the rabbits was explored by intradermal injections of India ink, it was found that the areas of spreading were, in general, inversely related to the survival time (390 mm<sup>2</sup> and 539 days in family A, and 934 mm<sup>2</sup> and 141 days in family F), but directly related to the volume of the local lesion in cmm (295 in family A, and 1059 in family F). Additional relations were established between resistance of the animal to infection (and consequently restricted spreading) and the following characteristics: (a) increased rate and intensity of antibody formation, (b) large volume of nodules resulting from successive intradermal injection of heat-killed tubercle bacilli, and (c) high allergic sensitivity in response to the latter treatment. The relationship between low skin permeability and each of the above listed manifestations of resistance was not an unvarying one, and discrepancies were found in some of the families of intermediate resistance, perhaps due to the lack of genetic uniformity. However, if one takes for each characteristic the most resistant family A, and compares it with representatives of two other families of intermediate and low resistance respectively, then a clear-cut relationship can be established.

*Sex and hormones* Again the most accurate data on sex differences are those of Lurie and Zappasodi (137). Quoting from one of their experiments in family A (resistant) the spreads in males were 441 mm<sup>2</sup> and in females 819 mm<sup>2</sup>, while in family F (susceptible) the spreads were 720 mm<sup>2</sup> in males and 1077 mm<sup>2</sup> in females. Comparable results were obtained in the other experiments. The effect of sex hormones was first studied by Sprunt, *et al* (235, 236).

Treatment during 26 days of young, castrated male rabbits with 1,000–2,000 daily units of theelin resulted in the reduction of India ink spreads from 555 mm<sup>2</sup> (control) to 237 mm<sup>2</sup> in one experiment and from 569 to 358 mm<sup>2</sup> in another. Incomplete treatment resulted in reduction of the spreads after 1 hour but not after 24 hours. In other experiments, groups of adult normal and castrated males and females were treated with alpha estradiol-dipro

pionate and the reduction of the spreads then were from 307, 324, and 336 mm<sup>2</sup> to 276, 279, and 289 mm<sup>2</sup>. Gonadotropic hormones were somewhat inconstant in their effects, and the positive results conflict with those to be reported below. However, factors depending on dosage and time could explain the discrepancies. The reduction of tissue permeability by estrone was paralleled by a reduction of the time of disappearance of the bleb formed by the intradermal injection of 0.2 ml of saline which took 129 min in the estrone-treated rabbits against 75 min in the controls. But most important was the fact that the susceptibility of the animals to vaccine virus injected intradermally was much lower in the estrone-treated rabbits with diminished skin permeability as compared with the controls.

Since corpus luteum hormones are known to be antagonistic to estrogens, Lurie and Zappasodi (138) studied the effect of the former on rabbits and found that they brought about a remarkable increase of tissue permeability.

Rabbits from inbred strains were given a single intravenous injection of 0.1 mg of gonadotropic hormone from human pregnancy urine. The spread of India ink in the skin, determined in each rabbit before and 2-5 days after the injection, increased in one experiment from 1064 to 1360 mm<sup>2</sup> in females and from 965 to 1188 mm<sup>2</sup> in males. The significance of the difference is appreciated from the fact that 75% of the female rabbits before hormone treatment spread the ink from 301 to 1200 mm<sup>2</sup> whereas after treatment 78% spread the ink from 1201 to 1840 mm<sup>2</sup>. Other experiments gave the same results, and in all of them besides the larger spreads, the amount of ink diffused, as judged by the blackening of the skin, was much larger in the treated animals than in the controls. A curious fact was that about 50 days after the hormone injection the permeability of the skin not only was not increased but was actually lower than before treatment (from 975 to 614 mm<sup>2</sup>) a phenomenon which may suggest a dual effect of the hormone and may perhaps explain discrepancies with Sprunt's work.

Weinstein (256) found that parathyroid hormone, and to a lesser extent extracts from the anterior portion of the pituitary, exerted a highly protective effect on mice against infections by *Bacillus anthracis*, *Escherichia coli* and *Pseudomonas pyocyaneus*, and also against tetanus toxin. The protection was as high as 75% in mice injected with 1,000 m.l.d. of *B. anthracis*. The effect of the hormones on tissue permeability was not tested in the infected mice, but spreading of India ink and testicle extract was much suppressed in rabbits similarly treated, this suppression being in direct relation to the protective effect.

*Individual and regional.* Wide individual variations of permeability in the skin of man or animals are common. Regional differences are quite clear in the rabbit skin, the tegument of the groins and axillae being much more permeable than that of the flanks. In human beings the permeability of the skin of legs to trypan blue is double that of the back (see 189), the opposite being found in rabbits (96) injected with pneumococcus autolysates. The ventral skin of guinea pigs is much more permeable to dyes than the dorsal skin (118). Despite its interest little is known about the permeability of the C.T. of different organs. We (65) have made numerous injections of 0.15 ml of India ink dilution into a variety of organs of adult rabbits and compared the resultant spontaneous and forced spreadings with similar tests in the skin of the same individual. It was tentatively concluded that the permeability of the C.T. in striated and smooth muscle, and in mesentery, pancreas, mammary and salivary glands, and lungs were about the same as in the skin, both spontaneous and forced spreading being practically absent. On the contrary, in the ovary and most pronouncedly in the testis both sorts of spreading were present.

*Functional and nutritional* McMaster and Parsons (199, 168) have shown that (a) spread of dyes is greater in the quiet living animal than in the animal just killed, (b) mechanical forces such as external pressure, motion, and pulsation promote spreading of the interstitial fluid, and (c) during the early stages of edema formation spread is rapid while in later stages the opposite is true. The immobilization of the limbs of rabbits (the experimental duplication of Trueta's method of treating war wounds) results in a very retarded absorption of snake venom and bacterial toxins (8), suggesting lowered permeability of the C T. The same effect was observed (232) in undernourished rabbits, and these animals showed a diminished susceptibility to vaccinal infection.

*Age* Numerous incidental observations (57) have suggested that the skin of young animals from several species is more permeable than that of the adults.

In purposely devised experiments, we found (65) that the average spontaneous and forced spreading of 0.50 ml of India ink dilution in the skin of 5 rabbits 1-2 years old was 5.8 and 7.0 cm<sup>2</sup> respectively whereas the same spreads in the skin of 20 of their progeny 25-60 days old was 10.8 and 12.6 cm<sup>2</sup> respectively. Some of the rabbits were killed and India ink dilution was injected into different organs to explore their permeability as it was done in the adults. The results were that both spontaneous and forced spreading was present in striated muscles just the same as in the skin, but absent in stomach, intestine and pancreas, suggesting that the C T of some organs can acquire a permeability of the adult type before that of other organs.

An exploration (97) of the permeability of young and old guinea pigs by intradermal injections of trypan blue showed an average spreading of 548 mm<sup>2</sup> in 69-day-old and of 284 mm<sup>2</sup> in 14-month-old animals. Increased permeability in young animals is always associated with softness but not necessarily with thinness of the skin. Simple caliper measurements of skin folds of rabbits in the above experiments (65) gave the same figures in mothers and progeny, and the experiments of Rocha Silva (216) further confirm the point. However, in guinea pigs, (119, 118) variations in thickness correspond to variations in permeability. The adult type of permeability in the skin is apparently acquired early in life, because no differences occur in the intradermal spreads in rabbits between 3 and 18 months (137).

It seems likely that besides constitution, sex, age, hormones, etc., tissue permeability is conditioned by other factors, and the first that come to mind are those vitamins (especially vitamin C) concerned with the building of intercellular matrices (257) and other substances which, as will be seen later, antagonize the effect of the S F's. It is beyond dispute that a permeable C T is an important factor in determining susceptibility to infection, and that we are entitled to extend to the whole organism that which has been learned from the study of the skin. The studies of Lurie were motivated (a) by the great differences in susceptibility to tuberculosis found among human races, (b) by the greater susceptibility shown by females to the same disease, and (c) by the critical stage of sensitivity to tubercular infection during puberty, which was experimentally reproduced by hormone injection. And in each case the experimental result was compatible with the hypothesis. The correlation was also strict with the results of Gottschall and Bunney who found it easier to infect young guinea pigs with tuberculosis than old ones. The results of Sprunt with vaccinia corroborate these findings.

Since the permeability of the C T is obviously not specific, it would follow that an animal with a high tissue permeability should have a higher susceptibility to practically any infection to which the species or the individual is naturally susceptible and *vice versa*. Sprunt has reviewed the clinical and experimental observations concerning the modification of various diseases by estrogenic hormones, and has added his own observations on the alteration of the course of myxomatosis in pregnant rabbits. But more convincing facts are found in the field of infection in relation to age. From our own experience we can assert that young hosts injected with the viruses of vaccinia (64), Shope fibroma (63), and Rous sarcoma (62) develop a disease which is both locally and generally much more severe than that shown by adults. Numerous other examples emphasizing the enhanced susceptibility of immature individuals can be cited (10). To be sure, other factors very important in infection and resistance are known to be absent or present respectively in individuals with a presumably high or low permeability of the C T. Many examples come to mind, but undoubtedly the most important among them is the low level in natural antibodies, and the difficulty of elaborating them under proper antigenic stimulation (both characteristics revealing an immaturity of plasma globulins) shown by young individuals (see 62, 10). Is there an unknown cause common to these factors and to the permeability of C T, or is there a possible cause-and-effect relation between some of them and the permeability of the C T? Tentative concepts in the latter direction have already been speculated upon by Lurie (136b), in considering the high production of antibodies to tuberculosis in rabbits with low skin permeability. Further support of the same hypothesis is found in the high antibody production in rabbits treated with estrone (100), and in diseased animals (see discussion of 3), in both of which a low tissue permeability can be presumed.

#### THE SPREADING FACTORS (S FS)

*Chronological* In 1928 we reported (49, 50) that the vaccinal infection of the rabbit was considerably enhanced when the virus was injected in the skin, along with aqueous extracts of rabbit, guinea pig and rat testicle. The effect was exerted on the host tissues and not on the virus because enhancement was also observed when an area of the skin was prepared by the extract several days before the virus was injected in the same area, and because the virus isolated from the enhanced lesions did not show any alteration in its infectivity. These observations were soon extended in our laboratory (69, 110, 201) to other infectious agents on several animal species, and duplicated in England by McClean (153). The spreading effect of extracts of testes, *i e*, the power to increase tissue permeability, was found independently by the latter author (153), and by Hoffman and Duran-Reynals (111, 112). Later, S FS were described from invasive bacteria (54), poisonous insects and snake venoms (61) and other sources.

Working along chemical lines Meyer, *et al* (179, 180, 186, 185) showed in 1936 that an enzyme present in autolysates of pneumococcus, tissue hash from rabbit

iris, ciliary body, and from spleen hydrolysed hyaluronic acid obtained from vitreous humor, synovial fluid, umbilical cord and streptococcus, this finding was duplicated in 1940 by Robertson, *et al* (214) with preparations from *Clostridium welchii* acting on synovial fluid. No correlation between all these enzymes and the S Fs was established until 1939 when Chain and Duthie (26, 27) in Oxford, identified some of the S Fs with mucolytic enzymes (hyaluronidases) and described the phenomenon of spreading in animal tissues as an enzymatic effect on the hyaluronic acid of the C T. The finding was soon confirmed and extended by several workers in both Europe and America.

*Distribution and varieties* Under the designation of S Fs, we include those substances present in living animal tissues which have the common property of increasing the permeability of the C T. Identification is based upon the ability of the substances to spread in animal tissues rather than upon their enzymatic activity *in vitro*, and one can state that variable amounts of S Fs are present in most if not in all normal and malignant tissues from many or all animal species. For some obscure reason they reach a high concentration in the testes of mammals and in poisonous secretions of certain animals. Testes of birds, amphibia and reptiles, however, contain them in only small amounts (17, 58, 65). Their presence in plants has not been reported. They are present in many bacteria, and reach high concentrations in certain invasive species. The filterable viruses so far studied are devoid of them. Despite incompleteness of studies and contradictory results on some of the properties of S Fs we shall tentatively group them as follows:

- (A) Factors with spreading power *in vivo* and an enzymatic effect on hyaluronic acid *in vitro* as shown by reduction of viscosity and by hydrolysis. They are those present in extracts of mammalian testes and sperm, spleen, skin, some eye tissues (no spreading effect studied in the latter) and in the secretions of certain invasive bacteria and poisonous animals.
- (B) Factors showing spreading power, but no enzymatic activity *in vitro* on hyaluronic acid. These are present in tissues other than those above mentioned and in some bacteria.

In still a third group one could include ascorbic acid, which has spreading power, a non-specific viscosity-lowering power, but no hydrolytic effect, which characteristics are shared by some other reducing substances, and possibly azoproteins. Meyer, *et al*, though, (178a) have not found any viscosity-lowering effect on pleural fluid by any of these compounds, and suggest that traces of enzyme present in the synovial fluid used as a substrate, but absent in the pleural fluid, could explain the discrepancies.

*S Fs of group A* A common characteristic of these factors is that they are the product of external secretion from the simplest organisms, bacteria, to the more complex. They serve either a nutritional or an aggressive function in insects, leeches, fishes, and snakes, or a reproductive function in mammals. Their properties were investigated by experiments on the living animal, with the factor from testis before its enzymatic properties were known (140, 142, 50, 112, 153, 154, 5, 82, 39), and more recently *in vitro* from experiments with factors

from many sources on preparations of hyaluronic acid (27, 109, 85, 86, 157, 162a, 163, 180-182, 214) The *in vitro* effect of the enzyme is determined by mixing the test material with either pure hyaluronic acid or a product rich in it and measuring after different periods of time, (a) the decrease in viscosity, (b) the changes in the precipitability of mucin by acetic acid, (c) the increase of reducing substances, and (d) the increase in N-acetylglucosamine (see 178a for criticisms on the latter test)

*Physical and chemical properties* These factors are very soluble in water, insoluble in most organic and many non-aqueous solvents tested, but soluble in glacial acetic acid and alcohol at certain concentration (140) Heating at 60° considerably or totally suppresses their activity, the inactivation being total at 100° They are rapidly inactivated by gentle shaking, and inhibited by chloroform but not by toluene, merthiolate, phenyl mercuric nitrate and acriflavine (27) Conflicting results have been reported on their inactivation or further reactivation by iodine, sulfites, arsenites, and cyanides (180, 27, 214) probably depending on the doses employed (178a) Powdered preparations may remain active for years In solution, they show a fair degree of stability between pH 4.6 and 9.0, but some streptococcus enzymes are unstable (178a) Some discrepancies have appeared concerning their destruction in highly acid or alkaline pH ranges (43, 140, 27) The factor in testis is inactivated by pepsin and trypsin but not by carboxypolypeptidase (154, 27) It gives the color test for proteins, and passes through Berkefeld filters, but not through semipermeable membranes which retain proteins, indicating that the factor has a high molecular weight, a conclusion not fully corroborated by ultracentrifugal studies (140) It is partly precipitated by many reagents commonly employed for protein removal, but it is not or only slightly precipitated by half-saturated ammonium sulphate (142) and by neutral lead acetate (188) Sodium flavianate, basic lead acetate or saturated ammonium sulphate precipitates it almost totally It is not affected by x-rays or ultraviolet light, in an electric field it migrates to the anode, in solutions between pH 6.2 and 6.9 (5) It is strongly absorbed by ortho aluminum hydroxide C and to a lesser extent by other adsorbents (141, 151) It does not lower surface tension (154, 142) It is inactivated by blood serum from several species (162)

On the basis of some of the properties above indicated several methods of purification have been devised (188, 82, 43, 151, 142, 141, 147, 38, 214, 27, 182, 144) Some of the preparations were still active on rabbit skin when diluted to 1:100,000, corresponding to the injection of 0.00005 mg substance (43) Concentration in aluminum thimbles results in preparations 0.5 ml of which spreads India ink over an area of 127 cm<sup>2</sup>, 55 times the area in the control (43) (See 140, 142, for criticisms on some of the purification methods)

*Characteristics of the reaction in vitro and in vivo* The degradation of the substrate by the enzyme *in vitro* occurs in at least three phases In the first, there is a destruction of clotting power with acetic acid (162a), in the second, there is a drop in viscosity without any free amino-sugar or reducing substances being detected, in the third phase, there is a progressive liberation of the latter substances, which reach a maximum in 24-48 hours At the end point, 70% of the theoretical reducing values have been obtained with some preparations (214) Special characteristics of some hyaluronidases have been detailed (107, 221, 178a), and different ways of splitting the carbohydrate linkages have been suggested (107) Conflicting results have been recorded (147, 148, 214) on the kinetics of the reaction with varying amounts of substrate or enzyme, but findings by McClean and Hale (163) concerning a lack of parallelism between variations of concentration and of viscosity of the substrate may provide an explanation

for the discrepancies. An interesting stoppage of the activity initially present in streptococcus enzyme has been reported (178a). The reaction takes place from pH 4 to 9, the optimum being from 5 to 6 with differences varying with the source of the enzyme (178a), and is profoundly modified by the salt concentration of the system (146, 154, 162a, 163, 214).

Although the tests *in vivo* may be more sensitive than those *in vitro*, the many variables conditioning the spreading in the rabbit skin preclude carrying out quantitative tests paralleling those *in vitro*. The spreading is most rapid during the first hour, and variations in the electrolyte content do not seem to affect it (81). Raising the temperature to 40° results in a marked enhancement of the spreading of testicular extract in guinea pig muscle (81). Generally speaking there is a direct relation between the size of the spreads in the skin and their degree of discoloration on the one hand and the amount of S F on the other, but this relation is far from being strictly quantitative. Nevertheless, Madinaveitia (140) has found the rate of increase of the spread to be proportional to the concentration of the factor during the first hour, becoming independent from it after 2 to 3 hours. If results from 3 rabbits are averaged it is possible to distinguish between the final spreads of tenfold dilutions of the factor. (See 72 for mathematical analysis of the phenomenon).

**Specificity** The spreading factor as an enzyme *in vitro* is perfectly specific. Substrates not attacked are polysaccharides from secretions of respiratory and digestive tract and from bacteria, also heparin, starch, mucoitin, and chondroitin sulfuric acid, etc., some of them very close chemically to hyaluronic acid (27, 86, 109, 163, 178a, 182, 214). Only hyaluronic acid or its sulfuric acid ester from mesodermal structures is attacked. Effects on some of the above substrates of bacterial preparations rich in hyaluronidase are due to the presence of other enzymes. Trypsin, taka diastase, lysozyme, emulsin, etc., are devoid of any effect on hyaluronic acid. On the other hand the C T of all mammals and birds so far studied can be permeated by the factors from testicle as well as by those from bacteria and animal poisons.

**SFs of group B** The grouping of these factors is justified, at least temporarily, by the lack of enzymatic activity on hyaluronic acid in preparations from tissues other than those of group A, and from certain bacteria which show a clear spreading power. The point is complicated by the fact that Cham and Duthie (27), who failed to detect enzymatic activity in extracts from 14 different tissues (including spleen and ciliary body) also failed to detect in them a spreading power despite the fact that such power seems undeniable. However, Hobby, *et al* (109) found that preparations from 5 bacterial strains, pigskin, and also hirudin show spreading but not enzymatic properties. (Rabbit skin, though, has been shown to contain hyaluronidase, 178a.)

Spreading power in organ extracts has been described by several authors, and it is difficult to explain the failure of Cham and Duthie to detect it unless they used too highly diluted extracts. Extracts of 1 or 2 parts of water and 1 part of the whole ground bodies of a variety of insects, mollusca, worms, and of individual organs other than testicle from amphibia, reptiles, birds (65, 17, 58),

and mammals (42, 44, 17, 229) show a clear although moderate and irregular spreading power, which in most cases vanishes after diluting the extract 5 or 10 times. There is no clear gradation in the activity of these extracts. Extracts of all tissues from leeches (39) and from the non-poisonous abdominal segments of scorpions (241) show a high spreading power, but there is the possibility that soiling by the active materials from neighboring secreting glands has occurred in the process of extraction. There is no increase of S F after anaphylactic shock or autolysis of some organs *in vitro* and *in vivo* (65). Extracts from castrated animal (42) and from fetal organs do not differ much from those of adults (17, 89). Blood serum is ineffective.

**Kallikrein** A substance present in "Padutin," a preparation claimed to be protein-free, obtained from human urine shows a very pronounced spreading power, 0.004 mg of the substance being active (31, 32). Enzymatic effects have not been investigated, and the substance is only tentatively included in the group. Kallikrein, the blood pressure lowering principle abundantly present in pancreas, salivary glands and urine, has been shown to be different from the S F from testes.

**Ascorbic acid** In view of the part played by this vitamin in the building of intercellular materials, the effect of ascorbic acid on tissue permeability is especially interesting. The effect of the vitamin on hyaluronic acid was suggested by the finding of Robertson, *et al* (214) that fresh vitreous humor of steers reduces the viscosity of synovial fluid. The authors suspected ascorbic acid (present in high amounts in the vitreous) as the responsible substance, an idea apparently confirmed by further work (215, 149, see 178a for criticisms). The vitamin is effective only in the presence of  $H_2O_2$ , and its effect is not limited to mesothelial mucins but extends to gastric and salivary mucins and to starch, pectin, flaxseed mucilage, cartilage, and pneumococcus capsules. The degradation involves a breakdown of the macromolecules without liberation of reducing substances or amino-sugars. Also, the kinetics of the reaction is entirely different from that of hyaluronidase (149, 163). McClean (163) discovered the spreading power of the vitamin, and reported that several other reducing substances, such as thiolacetic acid, pyrogallol, etc., shared the properties of vitamin C both *in vitro* and *in vivo*.

**Diazo compounds and azoproteins** The spreading effect of diazo compounds was found by Claude (37, 38) during the course of studies on purification of S Fs. Source materials such as sulfanilic acid, sodium nitrite, aniline were devoid of this effect. The spread of diazo compounds *e g*, p-diazobenzene sulfonic acid, is extremely rapid and the maximum is reached after a few hours. However, on coupling of diazo compounds with proteins such as horse serum, casein, egg albumin, atoxic preparations were obtained which spread more slowly than natural S Fs but the effect persisted for several hours, the final areas reached being often of more than 100 cm<sup>2</sup>. The protein acquires the spreading property upon coupling with an aromatic compound through a diazo group, but the entering aromatic radical fails to confer any specific character on the spreading phenomenon, and the type of protein selected for the reaction is immaterial.

provided it allows the formation of azo combinations. Azo-compounds of low molecular weight fail to exhibit any spreading power. Azoproteins are said to lower the viscosity of hyaluronic acid (27, 149) specifically (86, see 178a for criticisms), but no hydrolytic effect on the substrate is demonstrable (163), and the reaction is little affected by pH changes (86). Diazo compounds have an analogous viscosity-lowering effect (86, 149), and so has phenylhydrazine (149), but other carbonyl reagents are inactive.

*Other substances with spreading properties.* These are (a) substances derived from biological materials, such as some commercial peptones and lecithins (65), whose moderate spreading properties can probably be ascribed to the factors in tissues, and hyaluronic acid itself (109), (b) simple chemical compounds such as arsenious oxide (109), glycerol, and triacetin (65), (c) substances such as wintergreen oil which spreads or rather diffuses rapidly through the skin, oil of citronella (4) which induces an inflammatory spreading, and olive oil which shows only a forced spreading (65), and, (d) components other than hyaluronidase, present in some bacteria (16, 238) and venoms (141) showing special characteristics such as thermoresistance, slowness of action, etc. Little is known about the mechanisms involved in the different cases. No effect on hyaluronic acid has been reported or seems likely.

On the other hand, many substances have been unsuccessfully tested for spreading properties, among them adrenalin, histamine, saponin, bile salts, octyl alcohol, leukotactine, acetylcholine, tyrosine, dioxyphenylalanine.

*The reactions in vivo and in vitro.* The intercellular distribution of the materials injected together with S F as shown histologically, the lack of damage in the injected tissue, and above all the very characteristics of the unique phenomenon of spreading, all make it obvious that the effects of S Fs are exerted on the intercellular system of connective tissue, just as it is equally obvious that the effect, on whatever substrate exerted, is a rapid one. The nature and manner of action of hyaluronidases seem to fulfill these requirements, because these enzymes hydrolyze hyaluronic acid present in the ground substance of connective tissue, decrease its viscosity, and allow injected fluid to travel through the tissue. Moreover, the speed of the reaction *in vitro* is comparable to that of the spreading. Nevertheless, the explanation of the entire mechanism of the spreading on the basis of the enzymatic activity of the factors (27) has met with some opposition.

Data in favor are listed as follows. (A) All the S Fs from group A have both spreading and enzymatic properties, the latter being strictly limited to hyaluronic acid, while a variety of other materials lack both properties. (B) Azoproteins have spreading properties and lower the viscosity of hyaluronic acid (see 178a). (C) Heating of the factors results in a parallel suppression of both spreading and enzymatic effects. In addition, both properties are specifically suppressed in bacterial preparations by antisera (162a, 163), and inhibited by iodine (109). (D) Enhancement of both enzymic and spreading activity by inclusion of hyaluronic acid in culture medium (161).

Observations against the identification of S Fs and hyaluronidases are the following. (A) Lack of quantitative parallelism between spreading and enzymatic effects in the factors of group A (109, 145), combined with a suggestion (145) that it should be possible to separate

the factors responsible for both effects. However, the possible effect of different environmental conditions (pH, salt concentration etc.) in the skin and the test-tube needs to be pointed out (163). (B) Suppression of the enzymatic effect of the factor from pneumococcus by the specific antiserum without inhibition of the spreading power (109), although the limitations of the serum may require investigation (163). (C) Complete dissociation of spreading and enzymatic effect in the factors of group B (109).

On analysis of the problem, it is clear that although all the factors endowed with an enzymatic effect on hyaluronic acid have a spreading effect the reverse is not necessarily true. That a simple specific or non-specific reduction of viscosity of hyaluronic acid without hydrolysis is enough to allow spreading may be shown in the case of azoproteins, ascorbic acid, and some "depolymerizing" hyaluronidases (178a). That not even a detectable *in vitro* effect on hyaluronic acid is necessary is shown by the preparations studied by Hobby, *et al* (109), and probably several other substances have this property. These points pose the question as to whether hyaluronic acid can be affected *in vivo* by reactivated factors which do not show an effect *in vitro* (see 178a) or whether components of the connective tissue other than hyaluronic acid can be affected enzymatically or otherwise by these factors. The latter supposition, quite plausible on theoretical grounds, is made more probable by the existence of enzymes such as that described as attacking chondroitin from cartilage (see 214), that attacking heparin (see 97), that attacking collagen (see 141). (However, spreading preparations from streptococcus inactive on hyaluronic acid also failed to hydrolyse chondroitin-sulfuric acid, 173a.) On the other hand, although materials endowed with spreading properties seem numerous, it may well turn out on further study, that the responsible chemical radicals are common to many of the materials, and consequently the mechanisms of spreading involved will be quite restricted. The enzymatic specificity of the factors of group A and the specificity of the diazonium group in conferring spreading properties to different proteins support this idea. The possibility of releasing hyaluronidase there present or some other S Fs from the injected skin could perhaps account for the spreading properties of simple chemical substances (109) but direct proofs are lacking. Finally there still remains to be explained the spreading of inert mixtures in physiologically permeable connective tissue. Whether depolymerization and hydrolysis of hyaluronic acid result from the action of one or two enzymes has been discussed by Meyer, *et al* (178a). All this may serve to emphasize the complexity of the phenomenon of spreading and to justify the temporary grouping of the S Fs as we have done. (See discussion in 86, 163, 109, 27).

Differences in the rate of spreading, fast in the factors of group A, generally slow in those of group B and other spreading substances, may perhaps be taken as a biological basis for differentiating the S Fs with a hydrolytic effect on hyaluronic acid (group A) from the others. Rapidity of spreading may have no relation to the limit of activity of the preparation, and it is not possible to differentiate at the present moment between strength and concentration of the S Fs.

*Antigenic power and serological analysis* Because the S Fs have not yet been obtained in a pure state, there is still some doubt as to whether they are or are

not antigenic. The problem is of interest for the two following reasons (a) if they were highly antigenic, there would be cause to believe that the therapeutic effect of sera against invasive bacteria and poisons is, in part at least, due to its *antispreading* properties, and (b) if the S F's were not species-specific one could envisage the possibility of securing an *antispreading serum* effective against invasive infections and intoxications. It will be seen that if the latter possibility is not entirely supported by facts, it is very probable that direct or indirect neutralization of the S F's plays a part in the therapeutic effects of some antisera. It is a recognized fact (see 223) that other enzymes in the crystallized form are antigenic, hence passive protection against them is possible. This circumstance should be borne in mind while considering our problems.

We have shown (52) that rabbit antisera against crude or purified rat and bull testicular extract can be obtained which precipitate the extracts, suppress their spreading power and neutralize their enhancing power for vaccinal infection. McClean (156) obtained analogous antisera for preparations of *Clostridium welchii* with and without toxin, and later (163) showed that they suppress the hydrolysis of hyaluronic acid by the bacterial enzyme. Analogous antisera were obtained (61, 241) against snake venom, and against purified preparations of pneumococcus (109). Those obtained (194) against staphylococcus toxin presumably had antispreading effects. From the foregoing results, the following conclusions can be drawn.

(A) The antisera give a positive precipitin test, or in its absence a complement fixation test, for the immunizing materials.

(B) They not only suppress the enhancing effect of testicular extract on vaccinal infection, but the lesions induced by the mixtures of virus, extract and antiserum are actually smaller than those induced by the virus alone (52).<sup>2</sup>

(C) They suppress the spreading power of preparations from testicle (52), *C. welchii* and *Clostridium septicum* (156), snake venoms (61, 241) and staphylococcus (194). No suppression of the spreading effect of pneumococcus preparations has been obtained (109), although the enzymatic reaction was inhibited.

(D) They suppress the hydrolysis of hyaluronic acid by the enzymes present in preparations from *C. welchii*, *C. septicum* (163) and pneumococcus (109).

(E) Rabbits actively immunized against preparations from *C. welchii*, whether containing toxin or not, are protected against spreading induced by these preparations (156). The same holds true for rabbits actively (61) and passively (241) immunized against snake venom, but in the former case the spreading which is mostly affected by the immune state is the late spreading. This is said to be due to another component of the venom (141). On the other hand, rabbits immunized against testicular extract allow spreading and enhancement of vaccinal infection as well as normal rabbits (52). No anaphylactic reactions have been induced by purified preparations of testicular extract (156).

(F) The effect of each antiserum is limited to the preparation used for immunization. This holds true whether the precipitin reaction, the enhancement of the infection, the spreading power or the enzymatic effect is taken as an indicator of the antibody effect.

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<sup>2</sup> An analogous effect is obtained if vaccine virus is injected together with testicular extract heated at temperatures ranging from 60° to 80° (65). Partial but pronounced inactivation of the virus by reaction (adsorption?) with particles of the mixture or release of some inhibiting substance are possibilities to be considered. Heated extracts from other organs were practically inactive.

However, sera against streptococcal enzymes are group-specific (162a) One may add that the strength of the antisera is very moderate, the effect being quickly lost after dilution

Summarizing, there is a suppression by antisera of all the manifestations of the S Fs, but this effect is strictly specific The strict enzymatic specificity of hyaluronidases no doubt indicates a chemical identity of the substrates and may, at first thought, suggest the same for the factors themselves But in view of the results of serological tests one is forced to arrive at two alternative conclusions (a) The S Fs are antigenic but are radically different, in tissues or bacteria, even in closely related species This would indicate the presence in them of a common non-antigenic group which would determine the enzymatic specificity (b) The S Fs are not antigenic, and the suppression of their effects by antisera would be due to the incidental reaction with closely linked proteins which would inactivate the factors<sup>3</sup> In either alternative there would seem to be quantitative differences in the power to elicit antibody formation by the different spreading preparations as well as in the ability of the antibodies to prevent spreading in the immune animal

*The relationship between the S Fs and other components of the cell* Excepting in testicle, the S Fs from group A are constantly associated with toxic products Another possible exception is the enzyme from a soil bacterium isolated by Hirst (106) which induces rapid decapsulation of Group A streptococci In non-poisonous snakes (58), the spreading power of extracts from their supralabial glands is the same or only slightly above that of other tissues and has no toxic power On the other hand, in poisonous snakes, extracts from the washed gland tissue are almost free of both toxic and spreading power, but both factors appear together in the gland secretion This suggests the presence in the cell of an inactive precursor of the S F Similar cases of coexistence, in bacteria, of spreading and toxic effects will be described later The problem then arises as to whether the molecules of the toxic materials have spreading properties or whether S F and toxin are chemically independent but acting together as a unit While the situation may not be the same in the different S Fs some of the following observations decidedly favor the second alternative

McClean (156) obtained convincing evidence, in *C. welchii* preparations, that the S F is distinct from any specific toxin He secured antibodies neutralizing the spreading but not the toxic effect of the materials Moreover, the transformation from toxin to toxoid by the action of formaldehyde leaves the S F intact Madinavertia (143) showed in the same preparations, the independence of S F and proteolytic enzymes Claude (39) was undecided as to whether two distinct factors were responsible for the spreading and anticoagulating effects

<sup>3</sup> In some experiments (52), rabbits were immunized against testicular extract from the same species No flocculation could be obtained *in vitro* between their sera and the extract, but considerable suppression of the infection occurred when sera and virus were injected after incubation at 37° for 2 hours The strain of virus employed, Levaditi's neuro-virus, was maintained through testicular passages It may be that we were dealing with a neutralization of the S F still present in the diluted pulps containing the virus or with an effect analogous to the suppression of the Rous virus by sera against normal chicken tumors such as shown by Gye The problem calls for further work

of leech extracts, but Hirst (106) succeeded in separating hirudin from S F. In Russell's viper venom the S F is independent from crotoxin, the neurotoxic constituent, and from the blood-clotting component (141, 163). However, Favilli (84) having treated *Lachesis* and *Vipera* venom with different agents including formaldehyde, and found that the destruction of the toxin was accompanied by a parallel destruction of the S F, concluded with the suggestion that "both activities were inseparably linked to the same substance," while in our experiments (61) we concluded that "the relationship between S F and toxins of the snake venom is not yet understood."<sup>4</sup>

The S F in testes or sperm is not associated with any obviously toxic material, and it has been dissociated by chemical fractionation or other tests from enzymes active on  $\beta$ -glucosamine (70) and human cervical mucus (124), from Kallikrein (32), and from materials active on cancer cells, red cells, and ova of some species. Data on the effect of testicular extract on these cells follow.

We reported (51) that the injection of crude or partly purified preparations of T.E., from different species, mixed with cells of the Brown-Pierce tumor resulted in a pronounced suppression of the growth while serum was devoid of such effect. These results were duplicated by Tanzer (240) on some mouse tumors, but he found that T.E., highly purified was inactive whereas extracts heated at 60°-100°, still showed inhibiting power. It was, therefore, clear that contrary to what was first suspected (118) the S F was not the cause of the inhibition. Also, treatment with T.E. of mice bearing spontaneous or transplanted tumors resulted in failure (240, 65, 205). An analogous situation arose from the study of the effects of T.E. on red cells and ova of some lower animals. Favilli showed that treatment of these cells with T.E. greatly increased their fragility or permeability to water (75, 76, 46) and McClean had the same results (154, 155), but these effects were found lacking on repetition of the experiments (87) with highly purified extracts. Whether it is one or several substances that are responsible for the effects on each of the cells is not known.

Although no direct studies have been carried out on this point, it would seem logical to locate the S F of animal poisons in the protoplasm, and since the S F in testes is also present in sperm (112) this may suggest a nuclear origin. It is not known whether the S Fs from testes and other tissues can be released by mechanisms not impairing the cell or whether cellular disintegration is indispensable for this to take place.<sup>5</sup> It must be recalled in this connection that in at

<sup>4</sup> After tests on the effect of heat plus some other experiments to be taken up later, we concluded that "the two factors can be dissociated," the word "factor" being obviously used as synonymous with "effect." The primary object of the experiments was to bring proofs showing that the spreading was due to a "selective permeation of tissues by a specific factor," and not "to a sort of passive flooding of a highly damaged tissue by the venom components" (56) a point which although it might seem obvious had to be proved experimentally. Cases like that of *Clostridium histolyticum* inducing large destructive lesions and yet devoid of S F (156) had to be kept in mind. Heated venom induced lesions as negligible as those induced by other materials whose effects are undoubtedly due to the S F, yet the heated venom retained a spreading effect. That the latter effect was also impaired by the treatment was obvious from our data. These considerations answer some criticisms by Favilli (84).

<sup>5</sup> The culture of organs in the Lindbergh pump seem to provide a good opportunity to approach this interesting point. In the laboratory of Dr. A. Carrel we have repeatedly tested the spreading power of the nutrient solution before and after perfusing rabbit testicle

least some animals there is normally a disintegration of spermatozoa in the epididymus with subsequent reabsorption. That the factor from testes is linked with spermatogenesis is shown by the spreading activity of the sperm (112, 154) and inactivity of several endocrine preparations tested. Moreover, extracts from natural or experimentally induced cryptorchid testes from rabbits and rats spread much less than extracts from mature testes (112, 233, 65). Immature testes show little or no activity, the maximum content of S F in rat testes is found when these animals weigh about 90 gm, when testicular growth has reached its maximum, and to decrease later (187).

The possibility that the S F from testes and sperm plays a part in sexual phenomena suggests itself. It has been observed (124, 126) that human semen contains an enzyme capable of dissolving the cervical plug mucin, thus enabling the spermatozoa to penetrate into the uterus. However, this enzyme has been found to be different from hyaluronidase (27). On the other hand, there is a mucin-like substance (190) which accumulates in the sexual organs of female monkeys during the estrus phase, and this mucin, resembling that of synovial fluid, has been found to be hyaluronic acid (27). Preliminary studies (88a) have shown that the addition of mouse or rat testicular extract to mouse ova still surrounded by follicular cells results in the prompt disaggregation of the latter cells, presumably through an effect on the viscid cement present between them. The importance of this phenomenon in fertilization suggests itself. Extracts from several other organs were inactive. The possible role of hyaluronidase in ocular physiology has been pointed out (177).

The S Fs obtained either as a secretion or extracted by crushing the cells from group A, and probably some from group B appear to be present in the cells in very high amounts. Fresh snake venoms and testicular extract are usually active at dilutions higher than 1:1,000,000 and 1:100,000 respectively, 0.000015 ml of filtrate at *C. welchii* cultures is active (156) and extracts from leeches (39) are probably still more active.

*The spreading in the skin.* The phenomenon of spreading is unique. Immediately following the intradermal injection into rabbits, the choice experimental animal, of a material rich in S F plus India ink, the bleb flattens immediately and the inoculum spreads through the skin as fluid dropped on a blotter. The spreading persists, although at a diminished rate, during several hours. If forced spreading is tried it is amazing to watch how rapidly the inoculum can be dispersed in a few seconds over a large skin surface under small pressure, while a much stronger pressure fails to dislodge the bleb formed by the control mixture. If the rabbit is killed 24 hours after the injection one sees the ink has penetrated in the fascia and even into the muscle underlying the injected site,

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for several hours. In every case there was a release of S F into the solution, sometimes extremely pronounced. Nevertheless, final conclusions are prevented by the fact that histological examination of the testicle at the end of each experiment showed always a certain amount of damage in the seminiferous tubules. Whether this represents a normal phenomenon considerably exaggerated or is equivalent to an extraction of the factor by mechanical means cannot be decided.

and the regional lymph nodes appear blackened. However, unless exceedingly powerful preparations are injected a great deal of the ink is left around the point where the skin was pierced.<sup>6</sup>

Histological studies supply little information. After injection of T E, there is some derangement in the dermal collagenous bundles (153), an effect which can be ascribed perhaps to the action of the factor on the interfibrillar matrix, probably identical to the ground substance (11). If India ink is used as indicator one sees the scattered granules thinning out from the injected site and reaching far from it. Fibroblasts are sharply outlined by the ink particles, and if Prussian blue is used as indicator, one can detect intraprotoplasmatic granules (111). In the control injections practically all the ink is left in a small area around the point where the skin was pierced. Despite its interest, studies on changes in the metachromatic staining of the ground substance after injections of S.Fs are lacking.

*The spreading in other tissues* Favill (81) injected mixtures of India ink and T E into different tissues and found that spreading took place in striated muscle, fasciae, tendons, and gastric subserosal and subcutaneous C T as well as if not better than in skin. No spreading was observed in gastric smooth muscle (cattle) and in lung and spleen, a fact attributed to the characteristic of the reticular stroma in these organs. No conclusions were reached concerning spreading in hepatic, renal, and nervous tissue because the injected material was absorbed too quickly and no blebs were induced. In our experiments on the subject (65), we found that spreading occurred in striated muscle, and in about 25 different tests on rabbits we found that spreading took place in the stomach, intestine, and uterus. Whether spreading in muscle takes place along the C T there present or through the indifferent substance between fibrils cannot be decided. Spreading occurred in the mammary gland, pancreas, mesentery, ovary, and testicle itself while other organs could not be properly studied by the crude procedures employed.

The case of the cornea is interesting for it contains a sulfuric acid ester of hyaluronic acid (177) which gives a beautiful metachromatic stain (117), it also contains a hyaluronidase (177). Although some have failed to observe it (144, 209), spreading takes place in corneal tissue as shown by Braley and Meyer (17a) who injected the organ with the dye T 1824 mixed with hyaluronidase. The phenomenon is very slow. Moreover, the metachromatic reaction with toluidine blue disappears after treatment of the tissue by the enzyme.

There is no indication that the S.Fs permeate epithelia. T E does not pro-

<sup>6</sup> Another means of studying the influence of the S.F. from testes on tissue permeability consisted of measuring the speed of filtration of T E through tissue pulps as compared with saline, serum or other materials (65). In several tests with pancreas, subcutaneous connective tissue, and muscle, it was observed that T E always filtered through the pulp at a much faster rate than the control solutions. Discordant results were obtained only with rat muscle, while soft tissues such as spleen and liver from rats or rabbits did not lend themselves for these tests. No final conclusions are drawn.

Still another means of studying the effect of T E on C T consisted of "injections" of fibroblast colonies from tissue cultures with mixtures of T E and India ink with a fine capillary pipette. The impression was clearly gained that mixtures with T E spread through the tissue much faster than the control mixtures.

mote the intestinal absorption of foreign proteins (111), or of strychnine (91), or the cutaneous absorption of insulin (6), it does not enhance infections by certain neurotropic viruses following installation in the nasal cavity, nor does it enhance the infection by the virus of rabbit papilloma which exclusively attacks epithelial cells (65). It does not promote the penetration of drugs installed in the eye (217). Injected into the joint cavity, it markedly reduces the viscosity of the synovial fluid (207a).

*The effect of preparations containing S F's on capillary permeability* The intimacy of the connections between C T and blood and lymph vessels has already been pointed out. What will later be said concerning the common effects of certain hormones on the permeability of all these structures further emphasizes the analogies between them. Several indirect observations have shown that the permeability of the vascular system can be locally or generally increased by preparations rich in S F's.

These observations can be grouped as follows. (A) Local increase of the capillary permeability from without. Viruses (50) and dyes (112, 61, 6, 202) injected intravenously are promptly localized in the areas of skin injected with extracts of testicle, snake venom and invasive bacteria. Recent injury such as induced by shaving favors this localization (202), and in dogs, the localizing effect is more marked in very young animals (6). The edematous changes in the late phases of the spreading induced by powerful preparations of T E also suggest an effect on the permeability of blood vessels.

(B) Local increase of the permeability of blood vessels from within. A practically immediate preparation of the tissue for the occurrence of the Schwartzman phenomenon is obtained when the active bacterial filtrate mixed with T E is injected into the vascular system of the rabbit ear in which circulation has been temporarily stopped. Filtrate alone or mixed with a variety of other substances known to be active on blood vessels has no such effect, but local thermal hyperemia had the same effect as T E (225).

(C) General increase of capillary permeability from within. Preparation of the kidney for the Schwartzman phenomenon is obtained when the active bacterial filtrate mixed with T E is injected intravenously (226), and enhanced local spreading of India ink or of vaccinal infection (153, 61, 156) results when materials rich in S F are injected intravenously. The state of increased tissue permeability can persist as long as 3 weeks provided large amounts of T E have been injected (198).

Claude (39) speculated on the possibility that at least a part of the spreading is induced by a permeative effect of the S F on blood capillaries, and Rigdon (210) by macerating normal tissue in saline solution extracted (from skin, muscle, and testicle of normal rabbits) factors which injected intradermally localized trypan blue injected intravenously. These factors though were thermostable.

We have approached the problem directly (60) by studying in mice and guinea pigs the speed of passage of dyes from the blood into the tissues when the dyes were injected intravenously with or without T E. It was found that in the former case, the speed was very much increased as judged by the discoloration of the tissues. Only extracts of testicle were found active while fresh extracts from muscle, brain, and spleen, having little spreading power, were inactive. Purified preparations from testes with a great spreading power were specially effective. These experiments have been repeated on rabbits (6) with the same results. The factor from testes which increases capillary permeability does not alter the selectivity in the localization of dyes in tissues. Acid dyes, like Congo red or T 1824 which under normal conditions do not localize in the central nervous system, keep this property when injected together with T E. On the contrary, the normal staining capacity of Bismark brown, a basic dye, for the central nervous system (92) was much enhanced by a simultaneous injection of T E.

The effect of purified preparations of T E on the permeability of blood capillaries has been confirmed on rabbits in the ears of which a moat chamber permitted microscopic observations of the effect on the capillaries of extracts placed in the moat (16). When T.E. was placed in the moat, a striking increase of permeability took place to the extent that fluid passed through vessel walls, leaving the corpuscles and part of the plasma concentrated within the capillaries. When T 1824 was injected intravenously and T E was placed in the moat chamber the permeating effect on blood vessels was readily seen and within 13 minutes sufficient of the dye had passed out of the vessels to be readily visible in the tissues.

In view of all these facts, it would indeed be tempting to conclude that spreading in the skin and increase of capillary permeability are results of the same enzyme. However, recently Hyman and Chambers (29a) using a technic employing changes in the rate of edema formation could detect no appreciable effect of highly purified preparations of hyaluronidase, prepared by K Meyer, on capillary permeability. More work is required to ascertain whether the dissociation in the powers to increase tissue and capillary permeability applies to all the S Fs and whether some of them may have both powers. At any rate it would seem that the enzyme active on capillary permeability offers many functional analogies with hyaluronidases and is often if not constantly associated with them. Its physiological interest is obvious. Recent studies on capillary permeability (260, 30) emphasize the important role of the intercellular cement rather than the endothelial cell itself. "To the cell physiologist the extraordinary wide latitude of the permeability of the blood capillaries tends to preclude the possibility that this property is primarily that of living cells composing the membrane. Many substances to which living cells are impermeable readily pass through the capillary wall" (Zweifach, 260).

It is interesting to contrast the effect of T E on capillary permeability, first with its atoxicity when injected in large amounts into the blood stream of animals, and second with its ineffectiveness in producing any changes in blood pressure (32, 6). One may well add here that T E has no effect on the isolated guinea pig uterus and rabbit intestine (6).

It seems that the above said about blood capillaries applies also to lymphatic capillaries (167-169). That lymphatics are permeated by spreading materials injected intradermally is obvious, and the degree of discoloration of the regional lymph nodes by the indicator employed indicates the degree of permeation.

*The effect of the S Fs upon the rate of absorption of different substances.* McClean, et al (164, 165) have shown that the addition of T E to diphtheria antitoxin administered subcutaneously doubled its concentration in the blood in 2 and 5 hours after its injection, but no effect was detected when mixtures were injected intramuscularly or when T E was injected intravenously. The retarded absorption of antitoxin mixed with urethane (33) is presumably due to an inflammatory condition inducing opposite effects than those of the S F. The addition of T E to large amounts of saline injected subcutaneously, such as used clinically for clyses, results in a much accelerated absorption (219). The anesthetic effects of novocaine (65, 44) are also enhanced by T E. Permeation of endothelial tissue by T E is suggested by the presence in them of cements

analogous to that present in blood capillaries (260, 30), and by the increased absorption of some drugs injected into the peritoneum together with T E (218) All these results suggest possible practical applications of the S F, which have been discussed by Sanella (219)

*Factors conditioning the spreading in C T* It would seem that the spreading is a phenomenon in which vasomotor or other nervous reactions play no part This is suggested by the fact, first shown by McClean (154), that spreading also takes place, although to a lesser extent, in the dead animal and in isolated tissues 48 hours after excision (39, 61, 65, 81) No edema is found in these cases, which confirms the idea that in the living animal a permeating effect on blood capillaries takes place which may contribute to the final phase of the spreading However, devitalization of the skin such as induced by drying the tissue with organic solvents or a vacuum (154) to parchment-like dryness upsets the normal mechanisms regulating permeability

Three groups of factors concerned with (a) local injury and repair, (b) metabolic disturbances, and (c) hormonal effects, have a decided inhibiting action on the effect of the S Fs

(a) The effects of acute inflammation will be fully developed in a later section Spreading by hyaluronidases (65) and that brought about by oil of citronella (4) is poor or nil in areas recently injured or previously injected with India ink The degree of limitation increases with the time after the ink injection which results in the production and increase of scar tissue (4) The same is true for T E (44) and pneumococcus autolysate (95) in granulation tissue, but some penetration of T E and cocaine mixtures has been reported in cicatricial tissue of the eyelid (209) Goodner (96) has observed that spreading of pneumococcus autolysate is stopped by the "immunological barrier" created by infiltrating the skin with specific antiserum

(b) Ligation of the ureters in rabbits results in a pronounced diminution of the spread of T E injected in the skin 40 hours after the operation (189), an effect tentatively attributed to the new physicochemical conditions of the C T as a result of the accumulation of catabolites with subsequent hydrophilia of the tissue (83) That advanced tissue hydremia opposes spreading by T E was shown by the tests of Volterra and De Guli (254) on patients with edema from circulatory stasis or from kidney conditions, and by our experiments (65) in which mixtures of T E and India ink were injected intradermally into edematous extremities (of guinea pigs and rabbits) 24 hours after they had been ligated proximally The resultant spreads were always one-half or even smaller than those in the opposite healthy member

(c) The vaso-constrictive hormone of the posterior lobe of the hypophysis (vasopressine) injected either locally together with T E or snake venom, but also intravenously, considerably inhibits the spreading (83), an effect shared to a certain extent by adrenalin, while other substances having a dilating effect on vessels are inactive Again, the inhibition of the spreading has been explained on the ground of the tissue hydrophilia created by the hormones, an explanation supported by two facts first, that a certain time has to elapse after injection before the inhibition is manifest, and second, that inhibition by vasopressine does not occur in the excised skin of the rabbit where circulating fluid cannot be called in the interstitial system Parathyroid hormone and extracts of the anterior pituitary have been shown (256) to suppress to a marked degree the permeation of the skin by T E The experiments of Menkin (172) have shown that adrenal cortex extracts and desoxy corticosterone totally suppress the effect of T E and leukotoxin on blood capillaries The effect of the hormone on the spreading effect of T E, although it can be presumed, was not studied

In the above recorded results adequate tests have shown that hormones and metabolites do not inactivate S F's but make the tissue more resistant to penetration by them

#### THE SPREADING FACTORS IN INFECTION

*General Considerations* No matter how important a part the S F's may play in physiological phenomena it is through their effects on infection that they were discovered and it is in the field of pathology that their effects are best known. On reviewing these effects we shall try to explain them on the basis of the physiological and physico-chemical properties of the factors. Under experimental conditions, bacteria and viruses injected along with a S F are distributed through the C T probably more uniformly than India ink particles (55). All infectious agents which are pathogenic for or propagated through the C T, if injected in adequate amounts, have enhanced local, primary effects in tissues permeated by the S F from any source. The S F may be injected either along with the agents of infection or through other routes.

Thus, enhancement of the local dermal infection has been demonstrated (a) With about 50 different bacterial species or types by the S F's from testicle (69, 201, 108, 55), staphylococcus, streptococcus (54, 18), pneumococcus (96), snake venom (61) and azoproteins (38), as tested on the skin of rabbits, guinea pigs, rats and mice. The group includes bacteria of such varied pathogenicity, as the types of tubercle bacilli (255, 242) and chromogenic saprophytes (201), and with such diverse affinity for the skin in natural infections as staphylococcus and members of the typhoid-dysenteric group.

(b) In infections of the monkey's eye by a variety of bacteria associated in experimental infection with *Bacterium granulosis* (192), infection of the guinea pig skin by *Trichophyton cerebriforme* (74), and infection of rats with acid-fast bacilli isolated from patients with leprosy (131). T E was employed in all these cases.

(c) With 12 species or strains of filtrable virus by S F's from testicle (50, 55, 150, 159, 151), snake venom (61), staphylococcus (54), and by azoproteins (38, 40) tested on the skin of rabbits and guinea pigs. The group includes the viruses vaccinia (50, 150, 55, 93), herpes, vesicular stomatitis (110), rabbit fibroma (55), rabbit myxoma (213), Rous sarcoma (113, 66) and virus III (55).

(d) In infections of the central nervous system by the viruses of vaccinia, Borna disease (110) and herpes (102) injected intracerebrally along with T E. It is probable that the same holds true for the viruses of poliomyelitis (244), equine encephalomyelitis (114) and chorio-meningitis (102). The involvement of the meninges in these infections provides a good basis for explaining the enhancing effects.

Enhancement in all these cases was observed when the infectious agent, mixed with the preparation containing S F, was injected into normal animals. Revival of quiescent lesions followed the local application of S F from bacteriophage lysates (18) and nasal secretions (103), but not from T E (219).

In the following cases enhancement of the infection of susceptible animals by S F's was not observed. (A) Infections of the central nervous system by fixed rabies virus in guinea pigs (20), and equine encephalomyelitis (114) in guinea pigs and rabbits when T E was injected subcutaneously or intraperitoneally together with the virus, infection of rats and guinea pigs by the sub-maxillary gland virus injected intracerebrally together with T E (125), infection of the rabbit skin by the Shope papilloma virus (65) inoculated by scarification. The very special cell affinities, and mode of transmission of these viruses may explain the failures. Moreover, in the case of the first two viruses only clinical morbidity or

lethality were taken as indices of successfulness of the infections, so that differences in the involvement of the tissue could well have been missed

(B) In infection of guinea pig pads and of rabbit skin by certain strains of herpes and vesicular stomatitis viruses (207, 228), the reasons for this failure being obscure. It may be useful to mention that we, too, (52) had difficulty at first in showing enhancement of the Noguchi testicular strain of vaccinia by T.E., but later (55) such enhancement was easily proved

*The nature of the enhancing effect* Provided the amount of infectious agent injected is large enough, the S.F.s act on infection as a magnifying device and not as promoters of new conditions. No matter how pronounced is the enhancement of the infection the differences between the lesions induced by the infectious agents injected together with S.F.s and those induced by the agents alone are always of a quantitative order. In the few cases when an opposite impression might be gained by the apparent lack of lesions in the area injected with the agent alone, it can be assumed that the enhancement brought about by the S.F. is an indicator that infection, although extremely mild, also took place in the control area

Thus, vesicular stomatitis virus injected in the shaved abdominal skin of guinea pigs, a relatively refractory tissue, together with T.E. induced an extensive vesicular dermatitis, rich in exudate. Other areas of the skin of 5 of the same animals injected with virus alone showed no reaction, but 23 showed a pin-head size nodule. Experiments on rabbits gave similar results. A "weak" strain of herpes virus injected into rabbits together with T.E. induced severe lesions, often of 20 cm diameter. The virus alone induced no obvious lesions in some cases, but in most rabbits it induced small mild lesions (110). Injection of bacteria of low pathogenicity plus T.E. into the skin of mice resulted in rather extensive lesions. The same bacterial species alone failed to produce lesions in some animals, but in others induced mild lesions (200, 65).

On the other hand, the addition of S.F. to the inoculum has never succeeded in overcoming the natural resistance of animals to infection, or in altering the tissue affinity shown by viruses.

Thus, the injection of certain strains of poliomyelitis virus by several routes into rabbits (244) and rats (102) along with T.E., as well as avian tubercle bacilli to dogs (23), have not resulted in infection. No localization other than in the salivary glands was obtained when the virus of Kuttner and Cole was inoculated subcutaneously together with T.E. (125). However, according to Cerruti (25) the resistance of the dog to *Bacillus anthracis* infection can be overcome by T.E., the effect was shared to a certain extent by powdered charcoal besides extracts of other organs, so that complicating factors were present.

Generalization of the infection with death of the animal occurs frequently following enhancement of local lesions by the S.F. Thus, 25 per cent and 99 per cent of the rabbits infected with vaccinia and "weak" herpes virus respectively died of generalized infection (50). Similarly, a high proportion of mice injected with a variety of bacteria (201) died. Mortality in the control animals was nil or very low. Undoubtedly, the great increase of the infectious agent in the primary lesion is largely responsible for the generalization, but permeation of the lymph and blood vessels by T.E. probably plays a part in those cases where the process is very rapid and the primary lesion not very large (69, 200).

*The effect of organ extracts on infection* An analogous enhancing effect for infections by viruses (50, 65, 1) and bacteria (200, 65) is also shown by extracts

of some organs other than testicle, when injected together with the infectious organism in the skin

In this respect one can classify the organs in 3 groups (a) those which enhance infection, as kidney, liver, lung, placenta, and testicle, (b) those which do not modify infection, muscle, adrenal, brain, bone marrow, and (c) those that inhibit it such as spleen, but irregularity in these effects has been noticed. While the enhancing effects of organ extracts depend largely on their content in S F the inhibiting effect for some infectious agents shown by spleen extracts (50, 200) depends not on a decreasing of tissue permeability, but on some other factor, because these extracts are often endowed with spreading power. Also, extracts of melanotic tumors (68) strongly inhibit vaccinal infection and yet give large areas of spreading in the rabbit skin. Extracts of other transplantable mouse sarcomas and carcinomas of the mouse strongly suppress vaccinal and herpetic infections (249), and these tumors have been shown (17) to have large amounts of S T. The presence in these extracts of neutralizing substances for the infectious agents suggests itself. There is a complete suppression of the vaccinal infection by the antibodies present in T E from immune rabbits (50), these extracts being nevertheless endowed with a strong spreading power (65). Yet, that extracts of organs modify infection through additional effects besides changes in tissue permeability is indicated by those cases in which the amount of S F present in the organ seems insufficient to explain the magnitude of the effect obtained, e g, the enhancement of cholera infection in guinea pigs by a variety of tissue extracts (see 69), anthrax infection of dogs and thyroid extract (25). The work of Felton (90) on variations of virulence of pneumococci by transfers through media containing tissue extracts is important, lung media caused an increase in virulence while the opposite effect was obtained with spleen media. (See (161) for comparable experiments)

The examples given emphasize the complexity of factors extractable from tissues which intervene in infection and mask or distort the effects of the S F. However, in testicular infections the amount of factor present in the glands is so great that it overshadows, in many cases, any other conditioning factor. Several examples could be quoted, but the vaccinal infection of rabbits (237) is undoubtedly the best. When the criteria, (a) incidence of histo-pathological lesions, (b) virus content and (c) enhancing power of extracts for vaccinal infection on other rabbits, were followed to judge the degree of infection, it was found that of all organs studied testis alone yielded a high percentage of specific lesions, gave a high percentage of takes on sub-inoculation, and had a marked enhancing action. Moreover, intratesticular injection is the choice method of detecting the presence of small amounts of virus in infected tissues (Ohtawara, see 237, 1), and the same result is achieved by injecting extracts of these tissues together with T E (1). Moreover, intratesticular injections result in a more widespread generalization of the infection than injections through any other route.

#### THE BACTERIAL SPREADING FACTORS IN INFECTION

If the part played by the tissue S Fs in infection has many obscure points, this is not the case with the bacterial S Fs. The injection of cultures of bacteria secreting S F is attended by exactly the same phenomena as occur when the factor, from any source, is added to any infectious agent. Remembering the architecture of the connective tissue and the effects of hyaluronidases upon it, one feels that, at least the earliest phases of these experimental infections are

largely understood, and the same can be said for the fulminant conditions following some sorts of bacterial inoculation in man. On appraising the relative parts played by the S F and by other bacterial weapons in the invasion of tissues one has to bear in mind that the effects of the latter are directed against barriers (leucocytosis, formation of fibrin clot, etc.) which the invader itself has called forth, whereas the former acts on a preëxisting physiological barrier. This places the S F as probably the first weapon used by the bacterium against the organism.

*Staphylococcal and streptococcal infections* Possibly in no other case are the effects of the S F so well shown as in staphylococcus infection. Here the factor has been shown in all cases to have a strong effect on hyaluronic acid, and as best observed in rabbits, the size of the lesion following intradermal injection of cultures of the organism is largely if not entirely determined by the amount of the factor present in the inoculum. Introduction of this inoculum induces immediate spreading detectable by a blanching reaction.<sup>7</sup> As shown by us (54), in rabbits injected intradermally on one side with a given amount of culture, and on the other side with a bacteria-free extract of the same culture to which India ink has been added, the size of the lesions after 24 hours closely compare with the size of the ink spreads. Staphylococcus strains can be grouped (54) according to the quality and size of the lesions induced when injected intradermally into rabbits, that is by the amount of S F they secrete.

The part played by the S F in streptococcal infections is more complex. For one thing, S F (hyaluronidase) is present in some strains of streptococcus whereas its substrate (hyaluronic acid) is present in others in the capsule, thus duplicating in the infectious agent the same state of affairs as that present in different tissues of the host. The enzyme is unstable and rather erratic in its action (178a). Moreover, some strains have a S F which apparently is not hyaluronidase. It remains to be determined whether this substance is hyaluronic acid itself (109). Still other strains of some groups have capsules not composed of hyaluronic acid.

A definite although widely different spreading power was found by us (54) in bacterial extracts or broth cultures of 13 of 14 strains of streptococcus. In general, there was a fair agreement, although not so close as in staphylococcal infection, between the areas of the dermal lesions in rabbits and the areas of spreading of India ink injected together with bacterial extract. In other words, the degree of invasiveness was largely determined by the amount of S F. Good correspondence was found with two strains from erysipelas, an infection so highly suggesting the effect of the S F, and the same was found by McClean (163) in another 4 strains from the same disease. It is also known that other erysipelas strains induce large spreading lesions (212). However, lesions induced by some bovine strains were much larger than the spreads induced by their filtrates, and the speed of spread was low as compared to that of the erysipelas.

<sup>7</sup> The flattening of the wheal after intradermal injection of pathogenic staphylococcus cultures, and the appearance of a blanching zone, 1 or 2 hours later, spreading beyond the original wheal was already noticed in 1924 by J. Parker (54).

strains (65) The importance of the S F's in staphylococcal and streptococcal infections is further emphasized by the enhancement of homologous and heterologous infections by extracts from invasive strains, whereas extracts from non-invasive strains are entirely devoid of such effects

*Streptococcal hyaluronidase and capsule formation* In 1937, Kendall, *et al* (121) isolated hyaluronic acid from the capsules of Group A mucoid, hemolytic, streptococci, and this was later confirmed (107) Seastone (221) isolated the same compound from the capsules of Group C streptococci, and related the size and persistence of capsules in young cultures to this polysaccharide As Kendall and coworkers pointed out, this is the only known case, besides that of glycogen of the formation of the same polysaccharide by the organisms and by the animal body, this being perhaps the reason (Lancefield, 127) that it does not function either as an antigen or as a hapten See (221) for criticisms on opposite statements by Lowenthal (133)

McClellan (161, 159) has obtained interesting results from extensive studies on the respective parts played by capsules and hyaluronidases in streptococcus infection Among about 50 strains of streptococcus he found that 16 of them developed capsules Extracts of none of these spread in the rabbit skin or hydrolyzed hyaluronic acid Most of the non-capsulated strains either in their original form or after animal passage elaborated S F In most cases the capsules were composed of hyaluronic acid which was hydrolyzed by hyaluronidases from streptococcus or from other sources with the resulting destruction of the capsules, but in a few strains the capsules lacked this carbohydrate, and one of them itself secreted hyaluronidase Only 2 or 3 strains apparently lacked both capsule and hyaluronidase The inclusion of hyaluronidase in the medium prevented the appearance of capsules in the strains to which capsules were normal The capacity to form either capsules or hyaluronidase was not confined to members of one Lancefield group, and in Groups A and C strains were found showing either characteristic

McClellan concluded that capsules and hyaluronidase are mutually exclusive since the latter enzyme either would prevent the development of the capsule or would destroy it

Pradham (204) studied a Group C strain growing in a medium rich in serum and found that a thermolabile S F was detected only in 2-hour-old cultures when capsule formation was at its maximum, disappearing later together with the capsules From this he concluded that the S F would seem to be the capsular material evolved by the cocci in the very early stages of multiplication, and would be later destroyed or neutralized as growth proceeds This conclusion is contradicted by the work of McClellan (161) and Seastone (221) It is hard to find an explanation to the findings of Pradham It may well be that the spreading was due to a bacterial component other than hyaluronidase Seastone did not find any spreading power in the purified polysaccharide of a C strain, but such power although moderate was found in broth cultures of this strain (221, 222) Also, Blundell (16) induced slow, edematous spreadings with an acetone precipitate of a Group A streptococcus but, contrary to Pradham preparations, this power was retained after heating at 100° McClellan (159) found a trace of hyaluronidase in 20-hour-old cultures of capsulated strains, and he states that this provides a possible explanation for the early loss of capsules The mechanism of capsule loss as cultures age obviously calls for more work

The importance of the capsule in determining the virulence of streptococcus has been pointed out by several authors, and the correlation of hyaluronic acid with the capsules implicates this substance as a virulence factor. This has been proved to be true in Group C streptococcus (221). Strains highly virulent for guinea pigs produce much larger capsules and much more polysaccharide than do strains of low virulence. This has led to a curious and apparently paradoxical experiment by Hirst (107) who showed that mice and guinea pigs could be protected from intraperitoneal infections with a virulent Group C strain by repeated intraperitoneal injections of leech extract (devoid of hirudin). The protection was most probably due to the removal of the capsule *in vivo* since decapsulation of Group C strains (and of Group A strains as well) *in vitro* takes place almost instantly by the hyaluronidase of leech extract. Protection against infection by Group A strains by leech extract was only feeble, but Blundell (16) by using testicular extract has achieved better results in mice. However, these results have not been duplicated (159, 162) in other strains employing hyaluronidases other than those present in leeches. In view of the fact that the decapsulating effect lasted only a few hours, it was suggested by Hirst (107) that the enzyme was inactivated in the host. This was shown to be the case by McClean (162), who found that blood serum has a strong suppressing effect on hyaluronidase probably through competitive effects by substances similar to hyaluronic acid. (For antagonistic effects of serum and white cells on S F's see contributions of Tossatti and Graziadei in (44).) From the evidence at hand it would seem that hyaluronic acid in Group C streptococcus plays a major role in the virulence (Seastone uses the term "invasiveness") of these organisms while in Group A streptococcus substances other than the polysaccharide (the type-specific M protein, in the first place) are responsible for it.

*The relationship between S F and other staphylococcal and streptococcal constituents.* Facts, both clinical and experimental, suggesting an effect of the S F in these infections are numerous. Those reviewed by Okell (191) and of Angevine (2) illustrate the rapidity of invasion of tissues by streptococci which can be recovered from the regional lymph nodes in considerable numbers 15 minutes after intradermal injection. Little is known of the relation of the S F to other weapons of the invader, (leucocidin, fibrinolysins, ability to tolerate phagocytosis) and to the bactericidal power of the blood, prevention of formation of fibrin clot, etc. (194, 99, 15, 248, 104, 47, 245). The dermo-necrotizing effect shown by practically all *Staphylococcus aureus* strains (195) as the most immediate proof of their local invasiveness seems specially interesting in this respect. There is a constant association, however, between the production of S F and some of the cultural, chromogenic, and biochemical properties considered typical of *S. aureus*. Furthermore, experimental manipulations resulting in dissociation of *S. aureus* strains into other variants has resulted in corresponding fluctuations in the amounts of S F secreted (54).

*Pneumococcus.* The first studies suggesting the important part played by the S F in pneumococcal infection were those of Goodner (95, 96) who studied the lesions induced in rabbits by the intradermal injection of pneumococcus autolysates (dermal pneumonia). Large, spreading lesions were induced which

on histological analysis were found to duplicate the three classical stages of lobar pneumonia. Edema was the outstanding feature of the early lesions, and it preceded any significant change in the cellular picture (95). Infections induced in rabbits by pneumococci Type III of varying pathogenicity were greatly enhanced by autolysates from homologous or heterologous types (96). McClean (156) showed that filtrates of cultures from pneumococcus Type I had a pronounced spreading action and a hydrolytic effect on hyaluronic acid (163), whereas filtrates from cultures of the same strain after repeated passage through media containing specific antisera, which resulted in the loss of its pathogenic power, failed to spread. Meyer *et al* (178a) demonstrated the enzyme in all the strains tested including R and S forms of Types I, II, III and VI. The SSS has no spreading activity. These facts indicate that whatever explanation is put forward concerning the invasion of the pulmonary tissue in lobar pneumonia it should take into consideration the effect of the pneumococcal S F. Although lung is not a good tissue on which to detect spreading by direct injection, several integrating structures of the pulmonary tissue can presumably be permeated by the S Fs. The same can be said of the pleura, and the presence of hyaluronic acid in this tissue is suggested by its abundant secretion when the tissue becomes malignant. The increased amount of circulating glucosamine as shown in pneumonia patients by Nilsson (see 158) may be an indication of hyaluronidase activity.

The importance of the spreading peripheral edema in pulmonary invasion has been recently emphasized by Barry Wood (259) who quotes an observation of Sutliff and Friedeman (238) as of great theoretical interest. These authors have described a soluble substance in both rough and smooth pneumococci that causes a spreading edema in the skin and lungs of puppies. But this reaction was not observed in other animals, and the active substance withstood boiling for 2 hours. Goodner's studies are by far more illuminating, and the importance of the edema in tissue invasion was well pointed out by him. It may be that the edema-producing substance is different from hyaluronidase and, together with the anticoagulant factor also found by Goodner, and other weapons of the bacterium, plays an important part in pneumococcal infection.

*Gas gangrene infections* The study of the part played by the S Fs in these infections has been carried out entirely by McClean (156) and the statements that follow are selected from his paper.

*C. welchii* induces fulminating infections with marked general intoxication, the bacilli travel along the interstitial tissue of the muscle and are often found beyond the gangrenous area. All the strains examined elaborated large amounts of S F which was still detectable in filtrates diluted to 1:30,000. *Clostridium septicum* (*Vibrio septique*) also induces acute intense spreading edematous infections with swelling and induration of the tissues, some emphysema, and general intoxication. Filtrates from the 5 strains tested had significant but widely varying amounts of S F. *Clostridium chauvoei* varies considerably in its invasive power, and filtrates from its cultures also showed varying amounts of S F, but no correlation was found between the degree of invasiveness of the 6 individual strains and their spreading power. It must be said, though, that the latter property was investigated on rabbits while the former was studied on guinea pigs. *Clostridium oedematiens* (*C. novyi*) which according to Weinberg occurs along with *C. welchii* in gas gangrene infections

brings about a spreading gelatinous edema with marked general intoxication. However, the organism spreads far less rapidly through the affected part than *C welchii* does, and blood cultures during life are generally negative. Ten strains of the organism studied produced only traces of S F. *Clostridium histolyticum* is a powerful proteolytic and putrefactive anaerobe inducing intense local digestion of the tissues, but its invasive power is very slight. *Clostridium tetani* does not invade the tissues and can only initiate an infection under favorable conditions such as provided in mixed infections of wounds. Tests on several strains of both of the latter bacteria showed complete absence of S F.

In summary, one can state in general terms that those organisms which are characterized by their capacity for dramatic invasion elaborate S Fs. It was impossible to correlate variations of the factor with differences in the antigenic composition of filtrates.

*Diphtheria* McClean (156) studied many *gravis*, *intermedius*, and *mitis* strains grown in broth and found that many of them regardless of type produced S F in moderate amounts. Maximum production was detected after 1-2 days of growth. We also found S F in 2 strains examined, and there seemed to be a correlation between the size of the lesions in rabbits and the amount of S F (65).

O'Meara (193) has recently postulated that diphtheria toxin is composed of varying amounts of two substances. One of them is substance "B" which is lethal for the guinea pig and is predominant in the toxin as usually prepared. The other, substance "A," predominates in saline extracts from 48-hour-old cultures of *gravis* strains in Loeffler's medium. These extracts freed from bacteria do not contain the classical diphtheria toxin, but injected intracutaneously produce most extensive necrotizing and edematous lesions, which O'Meara regards as analogous to the S F, stating that proofs of their identity are accumulating. The part of this substance in infection would be to promote the invasion of tissues by the "B" substance. It is from the synergic effect of these two substances that the hypertoxic, invasive "bullneck" type of human diphtheria would result. In view of O'Meara's statements McClean (160) has reinvestigated the presence of S F in 50 diphtheria strains grown under a variety of media and has found that filtrates of only a few of them had spreading effect of moderate intensity and only one had a detectable enzymatic activity (See (71) for comment.)

*Combined infections* It would seem probable that the bacterial S F may offer an explanation of some puzzling clinical observations on combined infections. The case of streptococcal complications suggests itself first. Goodner (95) has reported an acceleration in the rate of spreading of the dermal lesions that occur when *Hemophilus influenzae* is used as an associative infective agent with the pneumococcus in the rabbit, whereas *H influenzae* injected alone induces only mild lesions. Shope (224) has speculated along similar lines in discussing the combined effects of *H influenzae-suis* and swine influenza virus. The possible effects of staphylococci on the activity of vaccine virus used for Jennerian prophylaxis have been frequently emphasized (see 247, 128). Some observations (208) suggest a possible effect of streptococcal S F in diphtheria infection and that of *C welchii* in spreading peritonitis (16a).

*Infections by other organisms, and by filterable viruses* Filtrates from *Strepto-*

*bacillus moniliformis* are endowed with a pronounced spreading power (still demonstrable at dilutions as high as 1 500), and also with an enzymatic activity against hyaluronic acid in extracts from Rous sarcoma. On the contrary, filtrates of the corresponding  $L_1$  forms were practically devoid of such activity (200)

In contrast with the invasive species just reviewed many other species, pathogenic or not, are devoid of S F. Among them are included all types of tubercle bacilli, the tested strains of meningococcus, *Eberthella typhosa*, *Rickettsia prowazekii*, *Pasteurella pestis*, and many others (54, 156, 65). It can, in general, be said that none of these bacteria induces large spreading lesions at the point of inoculation. Some other bacteria, though, such as mouse typhoid, and possibly a number of diphtheria strains (156) do produce extensive local lesions, and although we have detected some spreading power in autolysates of mouse typhoid cultures or in filtered extracts of the lesions they induce (65), it is doubtful whether the S F presumably present can explain the large size of the lesions, which (like those induced by some strains of streptococcus) spread for several days in the rabbit skin. Another problem is that of the anthrax infections where a spreading lesion appears only in the last phases of the disease. No S F was found in cultures of *B anthracis* (156), and it was doubtful if any was present in the edema fluid (65).

From many incidental observations on vaccinia, Rous sarcoma, rabbit fibroma and other virus diseases (65), the conclusion was reached that if the tissue which they infected did not contain S F a spreading effect was never detected in extracts from materials rich in those viruses.

In a direct approach to the problem (65), rabbits were injected intracerebrally with vaccine virus or invasive staphylococcus, and guinea pigs to the cornea with herpes virus. Extracts from the staphylococcus-infected brains induced pronounced spreads, but those from the brains infected with herpes or vaccinia, although having a high virus content, did not spread more than extracts from normal organs. Complementing the above observation, it was also found that purified vaccinia elementary bodies and extracts of cultures *in vitro* of the same virus were also devoid of spreading effect. Therefore, it can be concluded that the viruses studied are devoid of the property of secreting S F.

*S F in secretions and inflammatory exudates* It is not known whether the S Fs in these materials are secreted by tissues or are of bacterial origin but whatever the source the importance of these factors in infection seems to be obvious.

In 1931, Hanger (103) reported that in about 40 experiments, filtrates of nasal secretions from normal persons injected together with *Pasteurella leptoseptica* in the skin of rabbits induced a considerable enhancement of the infection which spread rapidly, and usually caused death in about 5 days. Secretions from acute colds were in general more effective, and the same occurred with nasal secretions from rabbits suffering from acute snuffles. We (65) have completed the above observations by showing that filtrates from 4 different nasal secretions during colds are endowed with spreading power. Hanger also reported that saliva showed the enhancing effect to a moderate degree, but tears were inert.

In experiments on rabbits (65) in which ligature of the intestine was carried out the peritoneal exudate showed a pronounced spreading power. Some of the exudates yielded no bacterial growth whereas abundant cultures were obtained from others. Filtrates of the intestinal content of rabbits showed a moderate spreading power. In experimental sta-

phylcoccal infection, serosal exudates containing the causative organism also contained large amounts of S F

*Fate of the bacterial and tissue S Fs during infection* When an invasive strain of staphylococcus is injected intratesticularly into rabbits an acute, fatal infection follows. The presence of S F in the blood can be demonstrated 3 hours after infection and continues throughout the life of the animal. India ink spreads considerably in the skin following intravenous injections, in rabbits, of staphylococcal filtrates, but the S F disappears very rapidly from the blood, suggesting elimination, neutralization or fixation in tissues (54)

In one experiment (65) rabbits were injected in one testicle with a strain of *Staphylococcus aureus* secreting large amounts of S F, and other rabbits were similarly injected with vaccine virus, an infectious agent not secreting S F. The animals were killed in successive days and the spreading power of extracts of normal and injected testes was studied. It was found that with the progress of the process there was in both infected testes a progressive diminution of the S F, until complete suppression occurred about the 6th to 8th day while the infectious agents were still present in the tissues.

These facts seem to indicate that in staphylococcus infection the circulating S F is mostly derived from the infected testicle and not from the bacterium. However, that there is a release of S F from dermal staphylococcal lesions can be suspected from the fact that the average area of spread of India ink alone in the skin of rabbits infected with invasive strains was larger than similar spreads in rabbits infected with non-invasive strains (54). Whatever its source, it is clear that the S F does not persist a long time in the blood stream, and we know that it is neutralized by blood serum (162).

The presence of S Fs in infectious exudates suggests an analogy with the so-called *agressins* described by Bail. Although some differences could be noted between the two factors, one has to remember that *agressins* are far from being well defined entities, and it is possible that on final analysis at least certain *agressin* extracts may be found to contain S Fs in sufficient amounts to account for some of the enhancement activity.

#### INVASIVENESS AND VIRULENCE

*The invasiveness of bacteria* Accepting the general term of *pathogenicity* as the power shown by some bacteria to implant themselves and multiply in the living tissues, it would seem warranted to group under a specific designation those among them secreting certain substances—of which the S Fs are the most important—primarily concerned with overrunning the tissues with which the bacteria first come in contact.

In 1933 (54), after finding that bacteria which are frankly virulent when introduced by certain routes do not show local invasiveness and do not elaborate S F, we inferred tentatively that *virulence* and *invasiveness* are not the same thing. McClean (156) has brought forward already reviewed experimental evidence on gas gangrene bacteria which "broadly speaking" confirmed the view, and recently (161, 159) has published data on streptococcus, of fundamental importance. Topley and Wilson (246), in discussing the confused terminology employed in the processes of infection, have suggested a distinction between invasiveness or the power "of rapid spread within the tissues" and virulence or the power "of bringing about the rapid death of a particular host" shown by bacteria. Okell (191) has

emphasized the same point in streptococcus infection "invasive and septicemic powers are not the same things", "nor is virulence as tested by the power to cause fatal blood infection in animals any guide to invasive potentialities in man" Menkin (170) after his studies of differences in the local inflammatory reaction (walling-off) induced by various bacteria has concluded that "virulence and invasiveness are therefore regarded as two separate variables"

That many bacteria are endowed with a high lethal power and yet do not induce spreading local lesions has already been mentioned (54) (156) Analogous dissociation is found in groups of the same species Thus, Rivers (212) described an erysipelas strain of hemolytic streptococcus which induced severe spreading lesions when injected intradermally in rabbits, but had no lethal power when injected intraperitoneally or intravenously in large doses Practically the opposite was found by Seastone (221) in his Group C strains, and Okell (191) mentions a strain isolated from a trivial wound that killed mice in intravenous doses of  $1/2,000,000$  ml Meyer *et al* (178a) found abundant hyaluronidase in R types of pneumococcus The same dissociation has also been observed between exotoxigenic power—an angle of virulence—and the induction of spreading local lesions Thus Mader and Halpern (139) after a study of more than 400 strains of diphtheria bacillus concluded that there is no relation between the size of the dermal lesions induced in the rabbit and the production of toxin, the best toxin-producing strains belonging to the group inducing the milder skin lesions The direct experimental approach of McClean (161, 159) has yielded the most illuminating data on the subject

He has shown that the invasive uncapsulated, hyaluronidase-producing strains of streptococcus have a very low virulence for mice after peritoneal injection while the capsulated A and C strains may or may not show a high virulence But when the invasive strains are injected intradermally their pathogenicity is greatly enhanced If injected by the same route into rabbits the invasive strains cause large spreading necrotic lesions with diffuse margins while the capsulated, virulent strains produce furuncle-like circumscribed lesions with little or no necrosis Complementing these observations, McClean has shown that passages through mice or through media containing hyaluronic acid result in a pronounced increase in the production of S F for the invasive strains, a response, as he points out, of probably great importance in the extension of infection The same procedure applied to virulent capsulated strains has failed to promote the formation of significant amounts of hyaluronidase McClean (163) had already determined that the addition of potassium hyaluronate increases the yield of hyaluronidase, thus giving experimental reality to a hypothesis of Robertson, *et al* (214) The same observations were also extended by McClean to pneumococcus Type I Goldie (94) has reported, an increase of the "local virulence" of diphtheria strains after passages through media containing organ extracts without any increase in the production of toxin

From these as well as from other experiments (54, 203), it follows that invasiveness, like virulence, can be specifically lost and recuperated by adequate experimental means, and in all probability the same happens in nature through bacterial variation

One can now approximately visualize the successive phases of the infection induced by invasive bacteria as follows

- (A) Hydrolysis of the mucoid ground substance of the connective tissue (a preexisting physiological obstacle), and multiplication
- (B) Spreading primarily through the *interstitial* system of the CT, now presumably a fluid medium, further multiplication with considerably increased secretion of hyaluronidase (159, 161), and further spreading Secondly, comparable effects are exerted from without on local blood and lymph vessels which may result in their invasion, subsequent dissemination of the infection, and secondary localization

- (C) As a result of the action of the S F's, and simultaneously, other characteristic bacterial components attack the barriers *called forth by the infection itself*, the most obvious barriers being phagocytes and fibrin

Changes in this sequence would depend on whether free S F is present in the inoculum as in experimental infections, on whether the S F was not hyaluronidase but some other bacterial component acting on C T or blood and lymph vessels, on whether S F's are liberated from tissues during the infection as in testicular infection, etc. Parallel phases in infection by virulent bacteria would be as follows

- (A) Multiplication, but not spreading, in the interstitial system with involvement of lymph and blood vessels
- (B) Dissemination by the circulating lymph, blood or both, and secondary localization

One could state that, in general, in lesions induced by invasive bacteria the portal of entry is always obvious and the propagation of the infection—spreading—is of an *active* nature, the direct result of adequate secretions of the invader, whereas in lesions induced by virulent bacteria the portal of entry is less or not obvious, and the propagation of the infection—dissemination—is by *passive* transport. It may be useful to remember in this connection how prominent the involvement of lymph nodes is in infections by virulent bacteria, of which tuberculosis is the best example. Such a lesion in early infection constitutes the "primary" clinical lesion, but its very location indicates a passive transport of the bacilli from an extra-lymphatic portal of entry. Examples have been quoted in which there seems to be a mutual exclusion between locally acting toxins linked to the S F and toxins of general effects, but generalizations would be risky. It seems clear, though, that within the complexity of each infection the presence of the S F gives a clinical community to conditions induced by the most different sorts of bacteria, the invasive bacteria

*The invasiveness of animal poisons* The same mechanism of tissue invasion by bacteria is also operative in many animal poisons. It has been shown (56, 61) that the venom of rattlesnakes (*Crotalus adamanteus*, *C. terrificus durissus*, *C. atrox*,) and water moccasin (*Agkistrodon piscivorus*) among *Viperidae crotalinae* contain very large amounts of S F. These venoms when injected intradermally into rabbits diffuse very rapidly and at the same time produce very severe local lesions. On the contrary the snake venoms from *Colubridae proteroglypha* studied proved to be frequently lethal, but those of cobra (*Naja tripudians*), death adder (*Acanthophis antarcticum*) and black tiger (*Notechis scutatus*) contained little S F, and the local lesions induced were comparatively mild while those of coral snake (*Elaps fulvius*) and superb snake (*Denisonia superba*) induced larger lesions, but never as severe as those of *Viperidae crotalinae*. The poison in the secretions of the skin glands of 6 species of toads behaved like snake poisons of the latter group in that they have little or no S F, and induce negligible lesions, but have a very pronounced lethal power. As a counterpart to these tests, extracts of the supralabial glands of harmless species of snakes such as the pine snake (*Pituophis cateniferis*) and chicken snake (*Elaphus quadrivittata*) contain very little or no S F and are entirely devoid of both local and general toxicity.

Confirming and extending these results Tarabini-Castellani (241) found a spreading effect in the venoms of *Vipera aspis*, *Lachesis alternatus*, *Lachesis lanceolatus*, *Crotalus terrificus*, and *Naja haje* employed at low dilutions, and titration of the *Vipera aspis* venom showed spreading at the dilution of 1:96,000. Also extracts of the whole venomous organs of the two poisonous fishes, *Trachinus vipera* and *Scorpaena porcus* showed spreading power,

although less pronounced than that present in snakes, but analogous extracts from two species of scorpions *Buthus bicolor* and *Euscorpis italicus* showed a high content of S F which, in the case of the latter species, was still evident at dilutions of 1:160,000 in extracts of the poisonous abdominal segment. Bruni (21) has indicated a rather pronounced spreading power in extracts of *Ankylostoma duodenalis*, which the author ascribes to the cephalic and cervical glands that elaborate the toxic, hemolytic secretion.

A state of affairs analogous to that present in species of the above zoological groups seems to be present also in insects, for it has been shown (56) that extracts of the whole bodies of spiders, bees, wasps and mosquitoes have a rather pronounced spreading power and induce local lesions of varying severity, spider extract being the most active, while extracts of non-poisonous insects are wholly inactive in these respects. Bee poison hydrolyzes hyaluronic acid (27).

It is clear that the presence of S F in the secretions of all these poisonous animals largely explains the rapidity in the development of the local and also general lesions following their bite or sting. The severe, edematous, necrotizing and hemorrhagic lesions following inoculation with rattlesnake venoms are the exact counterpart of the accidents following snake-bite in man. Tissue damage by some snake venoms is such that proofs were required to show that spreading was due to a selective permeation of tissues and not a simple diffusion through tissues whose texture and cohesion were so altered. By injecting rattlesnake venom intravenously and India ink intradermally or by injecting a mixture of both materials in the skin of dead rabbits, it was possible to obtain the spreading effect without any accompanying lesion (61). (See other experiments on the subject in footnote 4.) The content in S F is high in venoms of the *Viperidae* (rattlesnake) family, and relatively low in the *Colubridae proteroglypha* (cobra) family, and one could well speculate on extending to these venoms the same principles that we have applied to invasive and virulent bacteria. There is in some cases a mutually exclusive effect between the local and general effects of the venoms studied, as in the case of bacteria.

Claude (39) has shown that extracts of leeches have a very high content in S F which has the same distribution in the body of the animal as the anticoagulating factor, both originating for the most part in the anterior digestive tract, and it is conceivable that the S F is released together with the anticoagulant factor at the moment of the bite. Claude points out that the therapeutic application of leeches is sometimes attended by extensive suffusion of blood in the subcutaneous tissue.

*The invasiveness of malignant cells.* The presence of S Fs in extracts of many of the several animal and 46 human tumors studied has been reported (68), and extensive study of several tumors of rats, mice and chickens has disclosed a parallelism between the rate of growth and the amount of S F (17). It is curious to notice that Rous sarcoma apparently does not contain any S F while Fuginami's sarcoma does contain it. The former tumor—and other fowl tumors as well—secretes large amounts of hyaluronic acid (41, 120), but no data are available on the latter tumor which is just as viscid as the former. No hyaluronidase activity was found in the Jensen rat sarcoma (27). It is possible that one may have here a state of affairs analogous to that present in streptococcus involving a mutual exclusion of enzyme and substrate, and the problem is worth further investigation. The findings of Boyland and McClean suggested to them that the S F may control tumor growth. "It is possible that the increase of a transplantable tumor is limited by the amount of nutrient material which diffuses into it and that any substance which helps the diffusion may enhance the rate of growth. Alternatively, the presence of the diffusing factor may increase the permeability of the tissues of the host and so facilitate the growth of tumor" (17). It is known that cancer is the most permeable tissue to blood-transported dyes and sera (59).

#### THE "CRITICAL CONCENTRATION" OF THE PATHOGENIC AGENT IN INFECTIONS AND IN TOXIC PHENOMENA

*Bacterial infection.* This concept was evolved from studies in our laboratory in which the S F was employed as a tool for studying the effects of the local

dispersion of the infectious agent as a factor in resistance to infection (53, 55) The results obtained in bacteria can be summarized as follows

(A) The mild lesions induced by bacteria of low pathogenicity (such as *Staphylococcus albus*, *E. coli*, *Proteus vulgaris*, *pneumococcus R*, etc.), had their infections enhanced by T E only when the bacteria were injected in very high amounts, complete suppression by spreading being observed at lower concentrations

(B) The severe lesions induced by bacteria of medium or high pathogenicity (such as streptococci or mouse typhoid bacillus) had their infections enhanced by T E when the bacteria were injected in amounts varying according to the species or strain, but always higher than those of group (A) Partial or total suppression by T E was observed at varying levels below that concentration

(C) The very severe lesions induced by bacteria of very high pathogenicity, such as pneumococcus Type I (Neufeld strain 143), or human or bovine tubercle bacilli (242), had their infections enhanced by T E at all dilutions tested, the last ones theoretically containing one or a few bacteria \*

(D) If the experiment on pneumococcus was repeated on a rabbit that had had even small amounts of specific antiserum then suppression of the infection induced by large amounts of bacteria was easily accomplished by T E Control lesions induced by the higher concentrations of bacteria were not affected by the serum injected (see Fig. I in Ref. 55) †

From the findings it is reasonable to assume that enhancement of bacterial lesions will be observed, first when the concentration of infectious agent at the site of inoculation is so high that the scattering effect of dispersion fails to reduce the infectious agent below a *critical concentration*, and second, when the pathogenic power of the injected bacteria is so great that local agencies are insufficient to inactivate them even when widely separated in the tissues Inhibition will be observed when the concentration of bacteria at the site of inoculation is such that the diluting effect of dispersion will reduce it below the critical concentration, and when the pathogenic power is so low that the local protective agencies are able to inactivate the bacteria even when their number is relatively considerable

The concept of critical concentration adds a new angle to the relativity of the pathogenic power of bacteria, an effect always conditioned by the variable resistance of the host The example of infection by a strain of pneumococcus known to be lethal when a single bacterium is injected is specially illuminating by dispersing large amounts of bacteria through the tissues rendered slightly

\* It is interesting to notice that avirulent strains of acid-fast bacilli, for instance B C G and *Mycobacterium phlei*, behaved like the virulent ones when they were spread by T E The lesions induced by some of these avirulent strains were so mild that had they been induced by a non-acid-fast strain would have been most easily suppressed by spreading However, since the lesions induced by all the strains of acid-fast bacilli killed by heat are equally enhanced at all dilutions by spreading in normal or tuberculous animals, it is obvious that we are not dealing with an effect of virulence proper but with one of toxicity which depends largely on the fat constituents of the bacillus, because when large amounts of defatted bacilli were injected with T E, enhanced lesions resulted, but when smaller quantities were injected, suppression of the lesions occurred

† A similar experiment attempted with an anti-tuberculous serum and one human and one bovine strain of tubercle bacillus gave negative results This serum (No. 5807, Sharpe and Dohme) was almost entirely of antipolysaccharide character, had an antibody content very low if compared to antipneumococcus serum, and exerted a moderate suppressive effect only when the serum was injected into the skin together with the infective inoculum (243)

resistant by the specific antibody the high pathogenicity of the organism was reduced to nearly that of a saprophyte<sup>10</sup> Other striking examples were those of certain strains of streptococci which if mixed with saline gave lesions of 50 cm<sup>2</sup> or more, but repeated in such a way that each of the dilutions of streptococci (and staphylococci as well) mixed with T E were injected intradermally into one rabbit, and mixed with saline were similarly injected into another rabbit, the following paradoxical result could be obtained according to the dose injected and to the individual resistance of the host, the animals injected with bacteria suspended in T E developed no lesions and survived while those injected with bacteria suspended in saline developed large lesions and died

The same inhibition by excessive spreading was also observed when homologous or heterologous bacterial S F was substituted for T E an infection by a staphylococcus strain for instance was totally suppressed by injection of the bacteria mixed with filtrate from cultures of the same strain This suggests the existence of a delicate balance between the pathogenicity of an invasive strain and the amount of S F it secretes if bacterial inoculation is to be followed by successful infection Alteration in this balance must logically be a determinant in the infectivity of aging cultures where the amount of bacteria progressively diminishes while that of S F remains practically constant It may also conceivably play a part in the processes of natural or therapeutically induced recovery in a manner not dissimilar from the way the S F so greatly accentuates the effects of anti-pneumococcus serum Further evidence of the beneficial effect of dispersion on the process of infection has been brought forward in studies on a non-invasive infection, tuberculosis, by Thomas and Duran-Reynals (242) The data are summarized as follows

(a) While the addition of T E to human tubercle bacilli resulted in the most pronounced enhancement of both local and generalized lesions when the mixture was injected into normal guinea pigs, the repeated injection of T E into tuberculous animals had no untoward effect, even in an infection of recent origin (10 days) On the contrary, there was a slight difference in favor of the treated animals

(b) The resistance conferred upon tuberculous guinea pigs by superinfection was greatly increased when the bacilli employed were dispersed through the skin with T E The same treatment in rabbits was not followed by any extension of the new infection to the viscera

(c) The partial immunity conferred upon guinea pigs and rabbits by vaccination with heat-killed tubercle bacilli was increased as a result of dispersion of the vaccine through the skin with T E Also, tuberculous rabbits infected with dead tubercle bacilli suspended in T E showed an increased resistance to the disease when compared with controls receiving dead bacilli suspended in saline solution The addition of T E to tubercle bacilli injected into tubercular guinea pigs has been reported to counteract bacterial fixation by tissues (220), however, the dispersion did not result in any important change in the usual course of events following reinfection

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<sup>10</sup> As seen in text figure 1 and in table 4 of the Reference (196), the lesions induced by the last dilutions of the control suspensions of pneumococcus without T E are considerably smaller in the rabbits that received the antiserum than in those that did not receive it Since the volume of the inocula was constant and the bacteria therein in progressively smaller numbers, an effect equivalent to that of dispersion was obtained, and these control injections are themselves further confirmation of the experiment

*Virus infections* When progressively diminishing amounts of filterable virus were dispersed in the rabbit skin, the same as was done with bacteria, entirely different results were obtained

The experiments were carried out on 4 strains of vaccine virus, two of Shope, fibroma, and one of virus III (55), to which may be added three more strains of vaccinia (153) (231), one of Rous virus (113, 66), and probably two strains of herpes and one of vesicular stomatitis (200) It was found that the spreading effect of T E considerably enhanced the development of lesions due to these viruses in all dilutions employed, even those approaching the minimal infective dose If the experiment was repeated on a partly immunized animal, as it was done with pneumococcus, the following results were obtained if moderate amounts of serum were employed, there still was a moderate or pronounced enhancement by spreading according to whether the serum was injected intravenously or locally If larger doses of serum were injected, then an almost complete suppression of all lesions followed Suppression by dispersion as observed in the case of pneumococcus was never observed

In other words, the viruses studied were not suppressed in their action when spread throughout the tissues of the normal susceptible host, and consequently they behaved in this respect like very pathogenic bacteria, *e g* pneumococcus This held true for doses approaching the minimal infective dose (which in the case of pneumococcus we know may be a single bacterium) From this we concluded in 1935 that one may assume that the *critical concentration of virus per unit area of tissue is an infective unit of virus* (55, and personal communication in Ref 129 )

Doerr and Seidenberg and Keogh (see 196) after results from titrating vaccine virus on the rabbit skin and chorio-allantoic membrane of the chick respectively, suggested that a single virus particle could induce infection, and Parker and Rivers (see 198) showed that there was a high degree of correlation between the number of elementary bodies counted in a calibrated chamber and the number of lesions they induced Merrill (173) calculated that the minimum infective dose of vaccinia for the rabbit skin was at least 10 virus particles Parker (196), by applying appropriate statistical analysis (Poisson's law of small numbers) to the lesions following the intradermal inoculation into rabbits of suitably diluted virus suspensions, reached the conclusion that a single particle of vaccine virus was capable of inducing infection, and later he showed the same for myxoma virus (197) Parker's conclusions have promoted a controversy, and views favoring or opposing his have been brought forward Thus Smadel, *et al* (227) have obtained concordant results, and the same is true of Luria (134), who used bacteriophage in his studies, and analyzed statistically the results of others on animal and plant viruses Sprunt and McDearman (see 231) verified Parker's results, but thought the results indicated that one particle would infect if it reached a susceptible cell Sprunt, *et al* (234) suggested the possibility of reconciling their findings with Parker's if the factor of host susceptibility be taken into consideration Bryan and Beard (22) found very high values for the infectivity of the papilloma virus-protein, and attributed to the host the primary role in the determination of the pattern of response to papilloma, vaccinia, and myxoma viruses "contrary to Parker's concept that the character of the distribu-

tion is determined only by virus factors" Levaditi (129) studied the problem on vaccinia with the aid of fluorescence microscopy and found that the minimal number of particles capable of inducing infection was extremely variable depending on a number of factors of which tissue affinities of the virus, animal species, and natural or acquired resistance of the host would be unquestionable.

It is impossible to reach final conclusions from the data today available. It would seem though that at least a part of the controversy hinges around the concept of the *waste* of virus injected even into the perfectly susceptible host. Such waste is largely related to the number of susceptible cells available, and it may be presumably higher in papillomatosis,<sup>11</sup> where only epithelial cells are susceptible, than in vaccinia or myxomatosis where both epithelial and mesenchymal cells are susceptible. The great importance of the route of inoculation in this respect is illustrated by Levaditi's work (129). Waste would be minimal when the virus is dispersed as following injection into a highly permeable skin as in rabbits treated with S F, and maximal when the virus is concentrated as following injections into a skin of low permeability as in rabbits treated with estrone. This is what has been experimentally verified by Sprunt (231) who concluded that the chance of infection could not only be statistically predicted from the amount of virus (vaccinia) injected and the host resistance, but was also influenced by the number of cells exposed to each virus particle, in other words, by the *concentration* of the virus. (See (230) for other developments of the same idea.)

If one reasoned by analogy to what happens when bacteria are dispersed by the S F, several or many particles of virus of lesser pathogenicity would be required to induce a lesion. But, our experiments showed that enhancement of the infection resulted independently from the individual variations of susceptibility of the host and of the severity of the lesions usually induced by the virus, and Parker found that the single-unit rule applied to strains of vaccinia widely different in their pathogenicity. In other words, viruses always behave like bacteria of the highest pathogenicity so far as concerns their ability to implant themselves in the host but, contrary to these bacteria, this ability is independent of the gravity of the lesions that follow. Also, contrary to at least one of these bacteria, pneumococcus, dispersion of at least one virus, vaccinia, through the tissues of a partly immunized animal did not enhance at all the suppression of the virus by the antibody. It remains to be shown whether in this case the infectious unit was still a single particle endowed with special characteristics of infectivity or whether it was a complex of several particles acting together.<sup>12</sup> The S F, as an agent for dispersion, shows that virus infections of the skin are

<sup>11</sup> Dispersion of the papilloma virus through the interstitial system of the C T by means of the S F does not enhance the resultant infection at all (65).

<sup>12</sup> In connection with these results, it may be of some interest to record some studies on the effect of T E on neutral mixtures of vaccine virus and antiserum. Vieuchange (251) observed that rabbit T E added *in vitro* to mixtures of virus and serum neutral for the rabbit skin renders this mixture virulent for the same tissue, but the same experiment carried out by Fairbrother (73) did not result in any reactivation. The latter incubated his mixtures containing T E for 2 hours at room temperature, whereas Vieuchange incubated his for 30 minutes at 37 C and overnight in the icebox.

associated with local reactions different from those attending bacterial infections. Possibly the protection afforded by the association of the virus with cells may be a determinant of these differences, but inherent characteristics of the virus, the perfect virulent agent, seems to be the fundamental cause.

*Toxic and allergic phenomena* In 1930 McClean (153) reported the enhancement of diphtheria toxin by T E, and thereafter, the addition of this toxin to the inoculum as an indicator of spreading has been widely used. T E, though, was not found to enhance the effects of some enzymes and toxins injected in or under the skin (111). Bier (14) reported that the Schwartzman phenomenon was enhanced if T E was mixed with the toxin of the preparatory injection, while Cassuto (24) reported opposite results. Doerfler (48) found enhancement of the Koch phenomenon, but inhibition of the tuberculin and Arthus reactions. Analogous results were obtained by Bruni (19) with the latter reaction. In 1933 we (53) reported experiments on the reactions induced by the injections of a constant amount of T E and decreasing amounts of *E. coli*, toxins, and foreign sera, and on the Arthus and Schwartzman phenomena induced by the same materials. It was found that the dissemination of the infected materials by T E resulted in a considerable diminution in intensity of the induced lesion, and gains in size accompanied losses in severity. The milder reactions were completely suppressed by spreading.

However, the capacity for induction of systemic injury shown by the agents was not eventually modified by spreading. Thus, in agreement with Doerfler, the local tuberculin reaction in tuberculous guinea pigs was found to be reduced in intensity per unit area of tissue, but the toxic general effects were in no way altered (242). It is probably on account of differences in the permeability of the skin that one can interpret the results of Freund, duplicated by other workers (see 97), that older guinea pigs gave more pronounced skin reaction to tuberculin than young animals, but when tuberculin was injected intraperitoneally both age groups showed equal general susceptibility. However, Marabotti (see 88) saw the tuberculin reaction in human beings much diminished in presumably less permeable areas, and Joyner and Sabin (119) found no correlation between the intensity of the tuberculin reaction and the spread of a dye in the skin of individual adult rabbits, so that, in these cases other complicating factors were probably at play. As in the case of bacteria, the capacity to stand the suppressing effects of the spreading varied according to the strength of the toxic material and the degree of susceptibility of the normal or sensitized animal, and the concept of critical concentration, although modified by their incapacity for multiplication would seem to apply to toxic materials also. Some authors (48, 24) agree with this interpretation. An interesting aspect of the effect of spreading on toxic phenomena has been developed by Head and Thomas (105) in their study of burns. The inflammation brought about by such lesions, as induced by heat or cold in the rabbit skin, tended to remain sharply localized, but if T E was injected (locally in the lesion, or in the vein, or peritoneum), then a marked enhancement of the severe lesions, and a suppression of the mild ones, resulted.

The concept of critical concentration bears directly on the problem of the

number of infectious particles that must simultaneously reach the exposed individual to start infection, and suggests important modifications of the outcome of this infection by the degree of tissue permeability of the host. The same can be said concerning the local manifestations of phenomena of allergy and intoxication.

#### THE INFLUENCE ON INFECTION OF LOWERING OF TISSUE PERMEABILITY

*In inflammatory conditions* As a manifestation of inflammation, a mechanism is known which tends to protect the organism against infection in general, but more especially against the immediate effect of invasive bacteria. This mechanism is effective either in the inflammatory focus itself, or at a distance from it, and in both cases it consists of a lowering of the permeability of the tissues.

Favilli (77), in our laboratory, showed that treatment of the skin by intracutaneous injections of broth or staphylococcal filtrates previous to the injection of T E suppressed the spreading effect of the latter injected into the prepared areas. The same was found later for S F from *C. welchii* (88). In parallel experiments it was determined (77) that injections of living staphylococcus into areas of skin similarly prepared resulted in negligible lesions, whereas the same bacteria injected into non-prepared areas induced very large spreading abscesses. Later, the same observations were extended to vaccine virus cultivated in rabbit's testes (78). Taking as a working hypothesis that substances lowering cell permeability would antagonize the S Fs, Favilli (79) studied the effect of urethane and Ca salts and found that treatment of the skin with these substances resulted, too, in a considerable suppression of the spreading by T E, the same was also true for vaccinal (80) and staphylococcal (84) infections.

From these results Favilli first concluded (79) that the lowered permeability of the skin was the result of a direct effect on the permeability of the tissue cells. On restudying the question with McClean (88), it was found that all the materials suppressing spreading exerted their effects only when injected locally whereupon all elicited an inflammatory reaction. It was finally concluded that the lowered skin permeability was associated with the early stages of the inflammatory response.

A general alteration of tissue permeability in an infectious disease was first reported by Joyner and Sabin (118) who found that the spreading of Pontamine sky blue was much restricted in the skin of tuberculous guinea pigs. The average spread in allergic tuberculous animals was 1,214 mm<sup>2</sup> as compared with 2,102 mm<sup>2</sup> in normal animals. The same restriction was observed in guinea pigs affected with lymphadenitis due to streptococcus Type C, the average area of spreading being 1,359 mm<sup>2</sup>. Anergic animals in advanced stages of tuberculosis did not restrict the dye. The authors pointed out the opposition between the effects of tuberculosis and that of the S F and suggested that changes in the irritability and permeability of tissues were back of the phenomenon. These findings were duplicated in young tuberculous guinea pigs or in those injected with heat-killed bacilli by Gottschall and Bunney (97), but no differences were found between the skin permeability of old tubercular and old normal guinea pigs probably because, as already reviewed, tissue permeability is much lowered in old animals.

Duran-Reynals and Estrada (67) have shown that the induction in rabbits of even small lesions by invasive bacteria renders the whole skin impermeable which fact has a most important bearing on infection. A summary of the experimental basis follows

(a) When small amounts of staphylococcus or streptococcus cultures are injected intradermally into rabbits at intervals even as short as 30 min, there is a considerable and progressive diminution in the size and severity of the lesions resulting from the 2nd, 3rd, and 4th injections, these being from 4 to 50 times smaller than the first. The suppression is due to a non-specific and temporary lowering of the permeability of the skin, as shown by the fact that the spreading of India ink mixed with either saline solution or streptococcus filtrate is much restricted after the induction of the dermal lesions.

(b) The phenomenon is absent in the case of lesions induced by either bacteria of little virulence or killed by heat, but if S F from testicle or streptococcus is added to the former and large lesions result, then the phenomenon is shown again. It is also shown when large amounts of bacteria of moderate virulence are injected, but then it is never so pronounced as when S F is added to the inoculum.

Therefore, a pronounced lowering of the permeability of all dermal C T, and presumably of the C T of the whole organism, is brought about whenever the S F has contributed even to a comparatively mild infection, but since massive infections induced by virulent bacteria, without S F induce it too, it would seem that the S F, or the products of its action, is not the direct cause of the phenomenon, but the vector of another non-specific substance effective on the interstitial system of the C T. This hypothesis would be in line with the findings by Menkin (171) of chemically defined substances in the focus of inflammation responsible for one or another of its manifestations. Other possibilities have been discussed elsewhere (67). Despite the general increase of permeability which results from bacterial S F from staphylococcus injected intravenously, a transient increase, premonitory to the final decrease, in the permeability of the skin was never observed. However, it may be that in infections of long standing produced by invasive bacteria, the permeating effect of the S F dominates the situation. Thus, Volterra and DeGiuli (254) reported pronounced increase of dermal permeability in patients with pyodermitis or septic diseases with cutaneous localization. Facts reported by Rigdon (211) may bear some relation to this subject.

The lowering of tissue permeability at a distance from the inflammatory focus suggests that the occurrence of the same phenomenon in the focus itself is independent of the other acute local manifestations of inflammation, and the speed in the establishment of the general impermeabilization also suggests that local impermeabilization is the first mechanism antagonizing the local spread of infection.

It is not our purpose to discuss the part played by phagocytosis and other inflammatory mechanisms in opposing the steady spreading of lesions induced by invasive bacteria, and it is needless also to comment on the important contributions of Menkin (170, 171) in this respect. The phenomenon just described must be added to these mechanisms. Whether some of them are or are not

conditioned by the very early changes in tissue permeability remains to be ascertained

The lowering of tissue permeability as a result of inflammation throws light on two groups of facts. The first concerns the establishment of a resistance to infection 24 hours or less after treatment with a variety of bacterial preparations. The best known of these states is that described by Morgenroth, *et al* (see 67) on streptococcus under the misleading term "Depressionsimmunität". It seems likely that the data reported by Morgenroth have their basis in a general lowering of tissue permeability. The second group of facts are those reported by Besredka bearing on the "local immunity" of the skin after treatment with filtrates of old cultures of staphylococcus and streptococcus (antivirus). Favilli (77, 79) and Favilli and McClean (88, see also 13) have commented on the great importance that a lowered local tissue permeability, subsequent to the inflammation induced by the treatment, has on the genesis of this local resistance. Whether a diminished permeability inducing an increased resistance of the whole skin can evolve after such treatment remains to be determined.

*The mechanisms of lowering of tissue permeability.* Some of the effects reviewed regarding the influence on infection of physiological factors modifying tissue permeability are helpful in the discussion of the present subject. We can record the morphological changes brought about by some of these factors and try to correlate them with the resultant decrease of tissue permeability. Such reasoning has been used in trying to explain the beneficial effect of estrone treatment on gingival and vaginal infections, an effect attributed to morphological changes in the epithelia (see 236), but where the tissue permeability has been presumably diminished. The work of Loeb, *et al* (132) on mice, confirmed by Wolfe, *et al* on rats (258), has shown that repeated injections of estrogens bring about the intercellular deposition of a hyaline or fibrillar material which is collagen or its precursor, and the same phenomenon is observed with advancing age. The transformation of reticulum into collagen begins at a very early stage of life (5 days in rats), is largely completed in the younger rats, and continues more slowly in older animals. It seems very probable that estrone and some factor linked with aging has acted primarily on the mucoid ground substance hastening its transformation into argyrophilic fibers and collagen, and that this process has resulted in a more impervious intercellular system. But this would be no proof that the increased number of fibrils are responsible for its diminished permeability, because fibrils (McMaster) can well be conductors and not obstacles, and changes not detectable histologically may well be responsible for changes of permeability.

It can be surmised that most factors that antagonize the effect of S F's on the ground substance also diminish the permeability of the C T, and this has been shown to be the case with some of these factors. This suggests the possibility that quantitative or qualitative changes in compounds of hyaluronic acid are important factors conditioning tissue permeability. Experimental support of this hypothesis is found in the fact that hyaluronic acid accumulates in the

sexual skin of monkeys during the estrous cycle (190)<sup>13</sup> The great increase of synovial secretion, so rich in hyaluronic acid, in inflammation of the joints may suggest an analogous phenomenon in the inflamed C T, but nothing can be ventured as the cause of the diminished permeability of the C T away from the focus of infection

In summary, contrasting with what we have learned of the process of tissue permeabilization, very little is known of the opposite process Yet, it would seem that knowledge gained on the mechanism of tissue depermeabilization might lead to finding new means for the prevention and treatment of disease, and might explain effects of therapeutic agents hitherto not well understood

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<sup>13</sup> Chain and Duthie (27) also state that there seems to be an accumulation of hyaluronic acid in the skin in myxedema, but do not give the reasons justifying the statement

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